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American Journal of Veterinary RESEARCH

In this issue . . .

- Melanoblastic Tumors in the Dog 345
- Serum and Tissue Residues Following Selenium Injections in Sheep 422
- Metabolic Aspects of Ruminant Ketosis 496
- Erythrocyte Survival in Experimental Anaplasmosis 590

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CONTENTS ARE ON PAGES 337, 340, 342

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Contents

The articles in the Research Journal are grouped, loosely, by animal species, followed by the more general articles. The articles listed on the cover are those that appear at the beginning of the various divisions or are particularly representative of a number of similar articles in a division.

Melanoblastic Tumors in the Dog 345
R. M. Mulligan

Studies on Immunity to *Uncinaria stenocephala* Infection in the Dog—Double Vaccination with Irradiated Larvae 352
C. Dow, W. F. H. Jarrett, F. W. Jennings, W. I. M. McIntyre, W. Mulligan

Spontaneous Abnormal Cardiac Arrhythmias and Conduction Disturbances in the Dog—A Clinical and Pathologic Study of 3,000 Dogs 355
D. F. Patterson, D. K. Detweiler, K. Hubben, R. P. Botts

A Quantitative Measurement of the Blood Loss Caused by Ancylostomiasis in Dogs 370
C. H. Clark, J. M. Kling, C. H. Woodley, N. Sharp

Comparative Study of Feline Cytopathogenic Viruses and Feline Panleukopenia Virus 374
J. L. Bittle, J. B. Emery, C. J. York, J. K. McMillen

Pendulous Crop in the Chicken—A Pathologic Study 379
R. H. Rigdon

A Study of *Salmonella typhimurium* Infection in Market-Age Turkeys 382
R. Yamamoto, H. E. Adler, W. W. Sadler, G. F. Stewart

A Phage-Typing System for Staphylococci from Turkeys with Synovitis 388
W. W. Smith, G. A. James, M. L. Miner, E. Blommer, M. L. Jensen

Focal Necrosis in the Mucosa of the Gallbladder in Pigs with Hog Cholera 391
A. J. Luedke and H. W. Dunne

Application of the Conglutination Complement Absorption Test to Detect Hog Cholera Antibodies. I. The Technique 396
S. J. Millian and W. E. Englehard

Toxicity of Wood Preservatives for Swine 401
I. A. Schipper

Effects of Prenatal *Gamma* Radiation on Testicular Morphology and Function in Swine 406
H. B. Pace, R. L. Murphree, E. W. Hupp

Nutritional Muscular Dystrophy in Lambs—Importance of the Ewe's Diet at Certain Stages of Gestation and Lactation 412
S. Young, W. W. Hawkins, Jr., K. F. Swingle

Nutritional Muscular Dystrophy in Lambs—Administration of Selenium to Affected and Unaffected Lambs 416
S. Young, W. W. Hawkins, Jr., K. F. Swingle

(Continued on page 340)

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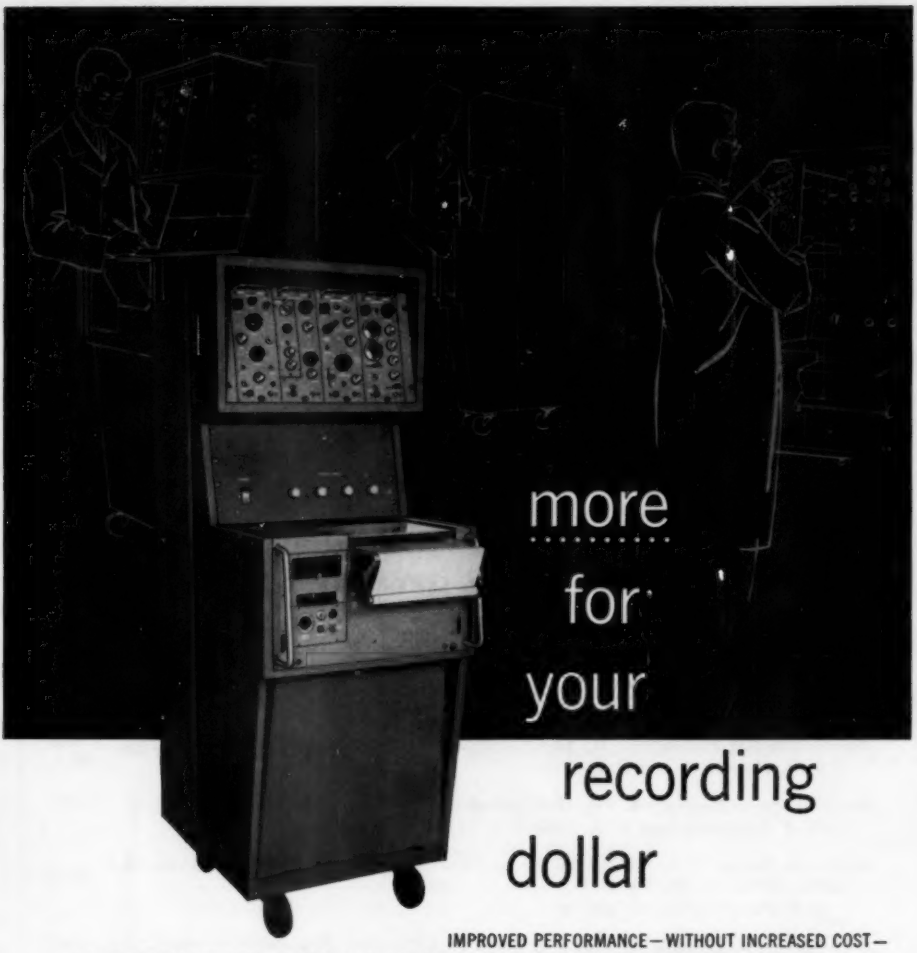

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(Continued from page 337)

Nutritional Muscular Dystrophy in Lambs—Effect of Administering Selenium to Pregnant Ewes 419
S. Young, W. W. Hawkins, Jr., K. F. Swingle

Serum and Tissue Residues Following Selenium Injections in Sheep 422
K. L. Kuttler, D. W. Marble, C. Blincoe

Enterotoxemia of Sheep. I. Effects of *Clostridium perfringens* Type D Toxin on the Brains of Sheep and Mice 429
L. A. Griner

Enterotoxemia of Sheep. II. Distribution of I^{131} Radioiodinated Serum Albumin in Brains of *Clostridium perfringens* Type D Intoxicated Lambs 443
L. A. Griner and W. D. Carlson

Enterotoxemia of Sheep. III. *Clostridium perfringens* Type D Antitoxin Titers of Normal, Nonvaccinated Lambs 447
L. A. Griner

The Isolation of *Vibrio bubulus* (Florent) from Sheep 449
B. D. Firehammer and S. A. Lovelace

Fecal Elimination of Estrogens by Cattle Treated with Diethylstilbestrol and Hexestrol 462
M. R. Callantine, M. Stob, F. N. Andrews

White Muscle Disease (Myopathy) in Lambs and Calves. VII. Etiology and Prophylaxis 466
O. H. Muth, J. R. Schubert, J. E. Oldfield

Studies on Shipping Fever of Cattle. II. Prevalance of Pasteurella Species in Nasal Secretions from Normal Calves and Calves with Shipping Fever 470
A. B. Hoerlein, S. P. Saxena, M. E. Mansfield

Bovine Papular Stomatitis. II. The Experimentally Produced Disease 473
R. A. Griesemer and C. R. Cole

Bovine Papular Stomatitis. III. Histopathology 482
R. A. Griesemer and C. R. Cole

Studies on Bovine Portal Blood. II. Blood Flow Determinations with Observations on Hemodilution in the Portal Vein 487
G. F. Fries and G. H. Conner

A Pasture Trial Using 2 Immunizing Doses of a Parasitic Bronchitis Vaccine 492
W. F. H. Jarrett, F. W. Jennings, W. I. M. McIntyre, W. Mulligan, N. C. C. Sharp

Metabolic Aspects of Ruminant Ketosis 496
D. S. Kronfeld

***Nocardia asteroides* as a Mammary Pathogen of Cattle. I. The Disease in Cattle and the Comparative Virulence of 5 Isolates** 502
A. C. Pier, M. J. Mejia, E. H. Willers

Foot-and-Mouth Disease Virus. I. Propagation of 3 Immunologic Types of Virus in Chicks 518
G. E. D. Zahran

Foot-and Mouth Disease Virus. II. Propagation and Modification of 3 Immunologic Types of Virus in Embryonating Chicken Eggs 527
G. E. D. Zahran

Foot-and-Mouth Disease Virus—Its Behavior in Cattle After Serial Passages in Chicken Embryos and Chicks 533
P. D. DeLay and H. Rosemeyer

(Continued on page 342)

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(Continued from page 340)

Effects of Chlorpromazine, Reserpine, and Ascorbic Acid in Resisting Heat Stress in Rats 537
T. Juszkiewicz

Effects of Chlorpromazine and Ascorbic Acid in Rats During Simulated Transportation Conditions at Normal and High Temperatures 544
T. Juszkiewicz and L. M. Jones

Thyroid-Active Agents and Heat Stress in the Rat 549
T. Juszkiewicz

The Effects of Chlorpromazine on Heat Stress in Pigs 553
T. Juszkiewicz and L. M. Jones

Studies on an Immunogenic Agent Against *Brucella abortus*. II. Preliminary Studies of an Immunologically Active Soluble Agglutinogen 558
R. C. Allen

A Heat-Inactivation Test for Differentiating Specific and Nonspecific Agglutination Reactions for Bovine Brucellosis 564
T. E. Amerault, C. A. Manthei, E. R. Goode, Jr., G. Lambert

Fluorescein-Labeled Antibody Technique for Identification of Leptospiral Serotypes. 570
W. G. Dacres

The Detection of Antibody Against *Leptospira pomona* in Composite Herd Milk Samples 573
E. A. Carbrey and R. A. Packer

Effect of Cortisone on *Leptospira pomona* Infection in the Guinea Pig 580
R. L. Schricker and L. E. Hanson

Estimation of Low Concentrations of Plasma Glucose Using Glucose Oxidase 587
L. A. Campbell and D. S. Kronfeld

Erythrocyte Survival in Experimental Anaplasmosis 590
N. F. Baker, J. W. Osebold, J. F. Christensen

Comparison of the Flotation of Metastrongylus and Ascaris Eggs in 3 Different Levitation Solutions 597
T. R. Bello

Effect of Naturally Occurring Highly Mineralized Water on the Growth of Laboratory Animals 601
B. H. Skold and R. Jensen

Metastrongylosis in the Field: Species and Sex Ratios of the Parasites, Preferential Location in Respiratory Apparatus of the Host, and Concomitant Lesions 606
S. A. Ewing and A. C. Todd

The Recovery of *Bacterium anitratum* (B5w) from Animals 610
E. M. Ellis

Scientific Notes 614

Cytologic Changes Induced by Infectious Canine Hepatitis Virus in Tissue Culture—*J. E. Prier* 614

The Effect of Urinary Silicon Concentration on the Formation of Siliceous Deposits on Bladder Implants in Steers—*R. F. Keeler and S. A. Lovelace* 617

Meetings 619

Research Tools 620

Correspondence 624

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Melanoblastic Tumors in the Dog

R. M. MULLIGAN, M.D.

Denver, Colorado

SINCE the report of 31 cases of melanoblastic tumors in dogs,[6] 57 new cases have been studied. Few, but important, reports on melanomas in the dog have appeared in the interim.[3,9] Since many new facets have been found in the analysis of the 88 cases observed in this laboratory, they are presented with regard to sex, age, and breed of the dogs; location and duration of tumors; and gross, microscopic, and follow-up findings. By this analysis, the following information pertaining to canine melanomas, not previously appreciated or of additive value, has been determined: (1) importance in prognosis of significant numbers of melanomas affecting both skin and mucous membrane with location in either of these sites; (2) sex differences related to the primary origin of melanomas in skin or mucous membrane; (3) age differences with relationship to noncancerous melanoma of the skin; (4) location and breed; (5) duration of melanoma before diagnosis in skin and mucous membrane; (6) size of melanomas of skin and mucous membrane; (7) the usefulness of size of a melanoma of the skin in determining whether the tumor is noncancerous or cancerous and thus predicting prognosis; (8) extension, recurrence, metastasis, and surgical salvage; (9) subdivision of noncancerous melanoma of skin and mucous membrane into types, depending on the location of the neoplastic melanoblasts; (10) origin of cancerous melanomas as related to the involvement of epithelium of skin and mucous membrane by neoplastic melanoblasts; (11) salient histopathologic features; and (12) 2 new cases of melanoma of the eye. Remarks will be included on the comparative pathology of melanomas in man and in the dog as appropriate under the preceding 12 points.

Received for publication July 8, 1960.
From the Department of Pathology, University of Colorado, School of Medicine, Denver.

MATERIALS AND METHODS

All tissues were fixed in 10% formalin and were usually accompanied into the laboratory by a slip bearing the date of removal; the names of the veterinarian and of the owner of the dog; the breed, age, and sex of the dog; the location of the tumor; and pertinent clinical details.

The tissues were described, appropriate pieces were processed into paraffin blocks by the dioxane technique, and sections 8 μ thick were routinely stained with hematoxylin and eosin. The descriptions of microscopic findings in the tumors were based on this method. Becker's silver nitrate method for melanin, the Turnbull blue reaction for iron pigment, and potassium permanganate-oxalic acid bleach were helpful in studying some tumors.

Definition of Terms.—"Noncancerous melanoma" is used to refer to a benign neoplasm of melanoblasts, ordinarily called "nevus," which has many meanings listed in any standard medical dictionary. "Cancerous melanoma" is employed to designate the malignant neoplasm of melanoblasts, often called correctly "malignant melanoma."

RESULTS

MELANOMA OF THE SKIN

Sex and Age.—The 58 cases of melanoma of the skin were observed in 33 intact male dogs, 20 intact female dogs, 4 spayed female dogs, and 1 dog of unknown sex; the male to female ratio was 1:4. The age distribution was as follows: 2 to 5 years, 4 dogs; 6 to 10 years, 28 dogs; 11 to 15 years, 25 dogs; and age unknown, 1 dog.

Location.—Of the 58 dogs, 57 had 1 site

involved by melanoma (29 cancerous and 28 noncancerous) and 1 had 2 sites involved (1 cancerous and 1 noncancerous). The 59 sites were as follows: head, 20; anterior extremities, 9; unspecified, 7; thorax, 6; abdomen, 4; posterior extremities, 4; anus, 3; neck, 3; and mammary gland, penile sheath, and tail, 1 each. Of the 20 melanomas of the head (14 noncancerous and 6 cancerous), 11 affected the eyelids. Of the melanomas of the anterior extremities, 1 was noncancerous and 8 were cancerous. At other sites, there was no special predisposition for cancerous or noncancerous melanoma. One of the dogs, in which the primary site was not specified, had 2 noncancerous melanomas. One of the dogs with melanoma of the abdomen had 2 primary noncancerous melanomas in this site.

Breed.—The following breeds were represented: Cocker Spaniel, 9; mixed breeding, 9; Scottish Terrier, 8; Boston Terrier, 4; Dachshund, 4; Airedale Terrier, 3; English Springer Spaniel, 3; Fox Terrier, 3; Collie, 2; German Shepherd Dog, 2; and American Water Spaniel, Belgium Sheepdog, Chihuahua, Chow Chow, Great Dane, Irish Setter, Irish Terrier, Irish Water Spaniel, Kerry Blue Terrier, Manchester Terrier, and Staffordshire Terrier, 1 each. One Airedale Terrier (surgically treated) with a noncancerous melanoma of the skin also had a cancerous melanoma of the mucous membrane.

Duration.—The stated duration of 15 noncancerous melanomas was 1 to 6 months in 9 dogs and 12 to 36 months in 6 dogs. The stated duration of 12 cancerous melanomas was 1 to 6 months in 7 dogs and 12 to 48 months in 5.

Gross Findings.—The noncancerous melanomas were firm to soft and dark to light brown, gray, or white. The cancerous melanomas were firm and dark to light brown, sometimes gray streaked or gray or white. The larger tumors were lobulated. The sizes of 27 noncancerous melanomas were: less than 1.0 cc. in 16 cases, 1.0 to 5.0 cc. in 9, 22.5 cc. in 1, and 52.5 cc. in 1; the last 2 were of hypodermal type (*see* "Microscopic Examination"). The sizes of 20 cancerous melanomas were: less than 1.0 cc. in 2, 1.0 to 9.0 cc. in 8, 10.0 to 100.0 cc. in 7, 101.0 to 200.0 cc. in 2, and 935.0 cc. in 1.

Follow-Up Examinations.—The tumors were obtained at necropsy in 9 dogs and at surgery in 49.

Of 15 dogs with noncancerous melanoma treated surgically, 5 without recurrence were euthanatized or died of other causes or metastasis at 7, 14, 17, 26, and 32 months after surgery. Of the remaining 10 dogs, 3 were alive and well at 3 to 12 months and 7 were well at 13 to 44 months.

Of the dogs with concerous melanomas, 5 had extensions of the primary tumors; 1 had a local recurrence; 12 had metastases available for study; 1 had clinical metastases; and 1 survived for several months after operation. The primary tumor extended into a bone of an extremity in 3 surgically treated dogs and into skeletal muscle of the head in 1. At necropsy in 1 dog, primary cancerous melanoma of the anus was found to extend into the rectum, prostate, and urinary bladder. In 1 surgically treated dog, the tumor was recurrent in an anterior extremity, the only example of recurrent cancerous melanoma of skin in this series. In this dog, in 5 other surgically treated dogs, and at 6 necropsies, primary tumors were recorded as follows: unspecified sites, 5; extremities, 4; and thorax, 3. Metastases were distributed in the lungs in 6, in regional lymph nodes in 4, in spleen in 3, in hypodermis in 2, in kidneys in 2, in liver in 2, in thoracic lymph nodes in 2, and in brain, heart, ileum, mesenteric lymph nodes, pancreas, pelvic lymph nodes, periaortic lymph nodes, peritoneum, and pleura in 1 each. In 3 surgically treated dogs, the time between surgery and necropsy was 2, 8, and 17 months. In 1 dog with cancerous melanoma of an eyelid, death from clinical metastases occurred at an unknown period after surgery. Only 1 surgically treated dog was alive and well 7 months after excision of a cancerous melanoma of the head.

Microscopic Examinations.—The ages at which 29 noncancerous melanomas occurred were as follows: 2 to 5 years, 2 tumors; 6 to 10 years, 16; 11 to 15 years, 10; and unknown age, 1. The ages at which the 30 cancerous melanomas occurred were as follows: 2 to 5 years, 2 tumors; 6 to 10 years, 12; and 11 to 15 years, 16.

The types of the 29 noncancerous melanomas were: dermal in 19 dogs, hypodermal in 6, and compound (involving epidermis and dermis) in 4. The 4 dogs with compound lesions were 6, 7, 8, and 10 years old. No example of pure epidermal involvement, epidermal melanoma ("junctional nevus"), was observed.

In the 30 cancerous melanomas, the frequency of involvement of the epidermis by anaplastic melanoblasts was as follows: epidermis ulcerated and residual epidermis involved, 4 of 15 tumors; epidermis not ulcerated but involved, 2 of 9 tumors; and primary lesion not available for study, 6 tumors. The incidence of infiltration of the epidermis by anaplastic melanoblasts might have been higher if this structure had not been ulcerated in 11 tumors and if the primary tumors of 6 dogs had been available for study.

The cells of noncancerous melanoma were polyhedral or short to long spindles. Sometimes, they had dendritic processes laden with fine melanin granules. The polyhedral cells were disposed in solid aggregates, in an alveolar pattern, or in small nodules. The spindle cells were arranged in bundles and occasionally surrounded small cutaneous nerves. The cells were closely approximated or overlapped, except when spread apart by edematous fluid or when in proximity to the inflamed or ulcerated surface. Whorls of cells resembled the structure of specialized nerve endings. The acidophilic cytoplasm was abundant in the polyhedral cells. The ratio between the volume of the relatively small, proportionate nuclei and that of the cytoplasm was reasonably constant. Nuclei were easily seen when melanin granules were few or lacking. When they were so numerous as to obscure nuclear detail, bleaching by potassium permanganate and oxalic acid was necessary to unmask the nuclei. When visible, the nearly always solitary nuclei were round, ovoid, or oblong, depending on whether the cells were polyhedral or spindle-shaped. Nuclei of a given shape varied little in size. The filamented, uniformly woven chromatin was regularly disposed within the nucleus around a single, discrete, small nucleolus. Rare, normal mitoses in the melanoblasts were most frequent in inflamed portions. The deep germinal cells of the usually intact suprajacent epidermis often contained fine melanin granules.

Cancerous melanomas consisted of polyhedral or short spindle cells inclosing variable, fine, melanin granules. Dendritic forms were rare. In well-preserved portions, the cells were loose and disposed in patterns like those in noncancerous melanomas. The cells were large, but the amount of cytoplasm was relatively small in relationship to the volume of the enlarged nuclei, which were round, ovoid, or, sometimes, gigantic and warped. Most of the coarsened, irregular chromatin localized at the edge of the nucleus with resultant thickening of the nuclear membrane. There were 1 to 3 large nucleoli; these often were in sharp contrast to a perinucleolar halo. Abnormal mitotic figures were common. Some tumor cells had a partly clear cytoplasm. Portions of tumor were sometimes necrotic or hemorrhagic. In the more anaplastic examples, melanin was usually lacking. The tumor invaded adipose tissue, skeletal muscle, lymphatics, and veins. The metastases had similar microscopic features.

MELANOMA OF MUCOUS MEMBRANE

Sex and Age.—The 29 melanomas of the mucous membrane were observed in 22 intact male dogs, 4 intact female dogs, and 3 spayed female dogs; the male to female ratio was 3:1. The age distribution was as follows: 6 to 10 years, 8 dogs; 11 to 15 years, 21 dogs.

Location.—The 29 melanomas (27 cancerous and 2 noncancerous) were located as follows: lip, 10 (upper, 3, lower, 7); gingiva, 6; cheek, 5; palate, 3; pharynx, 2; tongue, 2; and tonsil, 1.

Breed.—The following breeds were represented: Cocker Spaniel, 11; mixed breed, 5; Scottish Terrier, 3; Airedale Terrier, 2; Chow Chow, 2; Fox Terrier, 2; and Doberman Pinscher, English Setter, English Springer Spaniel, and Manchester Terrier, 1 each. As in melanomas of the skin, 1 Airedale Terrier (surgically treated) with a noncancerous melanoma of the skin also had a cancerous melanoma of mucous membrane.

Duration.—The stated duration of 1 noncancerous melanoma was 3 months. The stated duration of 19 cancerous melanomas was 1 to 6 months in 16 dogs and 12 months in 3.

Gross Findings.—The tumors were firm and dark to light brown, gray-brown, gray, or white. The size of 2 noncancerous melanomas was 0.2 and 1.5 cc. Because of the difficulty of obtaining accurate measurements of the primary tumor, the weights of 17 cancerous melanomas, with size available, were as follows: 2 to 9 Gm. in 8, 10 to 20 Gm. in 7, 21 Gm. in 1, and 32 Gm. in 1. Correlation of size with benignancy or malignancy could not be made in view

of the small sample of noncancerous melanomas.

Follow-Up Examinations.—The tumors were obtained at necropsy in 6 dogs and at surgery in 23.

The 2 dogs with noncancerous melanomas, 1 on the lower lip and 1 on the palate, were alive and well at 11 and 17 months after operation.

Of the dogs with cancerous melanomas, 7 had local recurrence, 3 had metastases available for study, 1 without recurrence was euthanatized, and 1 survived for many months after operation. Of the 7 dogs with local recurrence, 3 were euthanatized at 1, 1, and 7 months after operation, and 4 died at 1, 5, 6, and 9 months. In 3 dogs with metastases, the 1st (necropsy) had a primary tumor of a cheek metastatic to the brain; the 2nd (necropsy), of the palate metastatic to regional lymph nodes; and the 3rd (surgery), of the lower lip metastatic to regional lymph nodes. One dog with a primary tumor of a cheek, euthanatized at 15 months after operation, had no recurrence. Another dog with a primary tumor of a cheek was alive and well 44 months after operation.

Microscopic Examinations.—In 1 of the 2 noncancerous melanomas, the stratified squamous epithelium of the mucous membrane and the subepithelial connective tissue contained melanoblasts; in the other, only the subepithelial connective tissue was involved.

In the 27 cancerous melanomas, the frequency of involvement of the stratified squamous epithelium of the mucous membrane was as follows: epithelium ulcerated and residual epithelium involved, 6 of 18 tumors; epithelium not ulcerated but involved, 2 of 5 tumors; epithelium ulcerated and entirely absent, 4 tumors. The incidence of infiltration of the epithelium by anaplastic melanoblasts might have been higher if this structure had not been ulcerated in 12 tumors and completely destroyed in 4 tumors.

The microscopic features of noncancerous and cancerous melanomas of mucous membrane are, altogether, comparable to those described for melanoma of the skin.

MELANOMA OF THE EYE

Case 1.—A male German Shepherd Dog, 5½ years old, had a cancerous melanoma. This gray, red-mottled, 15-mm. tumor, affecting the anterior part of the choroid of the right eye, had been noticed for 3 months. The histologic structure was that of the so-called epithelioid type of cancerous melanoma. The dog was alive and well 16 months after enucleation of the involved eye.

Case 2.—A male Cocker Spaniel, 8 years old, had a cancerous melanoma. This soft, dark-brown tumor, excised from the corneoscleral junction of an eye, was observed for 5 months. The histologic features were similar to those described for cancerous melanoma of the skin. No follow-up information was obtained in this case.

DISCUSSION

Of the 59 melanomas of the skin in this series, 30 (about 50%) were cancerous. Of the 29 melanomas of mucous membrane, 27 (about 90%) were cancerous. These figures indicated that a dog with a melanoma of the mucous membrane has a 1.8 times greater chance of the neoplasm being cancerous than a dog with a melanoma of the skin. In man, the odds for melanoma of the skin becoming cancerous are much less[1] than in the dog due to the frequent removal of noncancerous melanoma for cosmetic or prophylactic reasons. Cancerous melanoma of mucous membrane is much less frequent in man than in the dog in comparison to cancerous melanoma of the skin.[2]

In this series of dogs with melanomas, the male to female ratio of 1.4:1.0 for melanoma of the skin is distinctly lower than that of 3.1:1.0 for melanoma of mucous membrane. Since a survey[7] of dogs licensed in Denver indicated a male to female ratio of 2.4:1.0, the assumption is reasonable that a given type of tumor has a significant predilection for the bitch, if the male to female ratio is 1.0:1.0 or less. Thus, melanoma of mucous membrane definitely favors male dogs, and melanoma of the skin is somewhat more common in males. No significant difference for sex was noticed for noncancerous melanoma of the skin as compared to cancerous melanoma of this structure. No sex preponderance for melanoma is found in man,[1,2,4] regardless of location or whether the tumor is noncancerous or cancerous.

In the skin of dogs, the peak incidence of noncancerous melanoma was at 6 to 10 years, but that of cancerous melanoma was at 11 to 15 years. The peak incidence of melanoma of mucous membrane was at 11

to 15 years, with 27 of the 29 primary tumors being malignant. The occurrence of cancerous melanoma of both skin and mucous membrane in the dog, in the advanced "cancer age" for this species (estimated as 6 years of age and beyond), parallels the known increasing frequency of cancerous melanoma in man at 40 years of age and beyond. Somewhat surprising in this series of melanomas in dogs was the paucity of melanoblastic tumors of the skin and the lack of them in mucous membrane in dogs under the age of 6 years. Certainly in man,[4] the congenital appearance of all types of noncancerous melanoma is so usual that such a phenomenon might be expected in the dog. If so, this was not evident in the series reported. Owners of dogs are prone to have the cutaneous tumors of their pets removed, as witness the large harvest of histiocytomas. Of 129 tumors studied in this laboratory, 112 appeared before the age of 5 years, 84 at 2 years of age or younger.

In man and dog, the tendency for noncancerous melanomas of the skin to be commonly located on the head and trunk is roughly comparable.[1,4] The posterior extremities of the dog were relatively less often affected by cancerous melanoma as compared to the high incidence in the lower extremities in man.[1,8] Noteworthy in this series were 8 cancerous melanomas of the anterior extremities. Also, only 1 example of melanoma, a noncancerous lesion, occurred on the genitalia.

Since the previous report,[6] the high incidence of melanoma in the moderately or heavily pigmented breeds has been confirmed. This contrasts with the occurrence of melanoma in man in light-skinned races and in blonds.[8] The frequency of melanoma in the Cocker Spaniel, 21 of 88 cases (24.0%), was striking and was not so evident before.[6] Most dogs of this breed had medium to dark-brown or black coats. The next most often affected breed, the Scottish Terrier, was represented in 11 of the 88 cases (12.5%). The black coat of most dogs of this breed is well known. Since these 2 breeds ranked 1st and 5th in a survey of dogs licensed in Denver,[7] the high frequency of melanoma in them may not be significant. However, the Fox Terrier, which has a light coat, ranked 2nd in this survey and accounted for 5 of the 88 cases of melanoma (5.6%).

The duration of noncancerous and cancerous melanoma of the skin did not vary significantly, with about the same number of cases recorded for 1 to 6 months as for 12 to 36 months. The duration of cancerous melanoma of mucous membrane was 1 to 6 months in about 80% of the dogs with such signs as drooling, oral fetor, and emaciation related to difficulty in mastication and deglutition.

By way of confirmation of previous observations,[6] 2 of 18 (11%) melanomas of the skin were less than 1 cc. and were cancerous; 2 of 12 (83%) melanomas of the skin were 10 cc. or larger and were cancerous, indicating the importance of size in 30 (64%) of the tumors with known size in detecting gross evidence of benignancy or malignancy. In the remaining 17 cases (36%), the size of the cancerous and noncancerous melanomas of the skin overlapped in the range of 1 to 9 cc. The size of melanomas of mucous membrane might have been valuable in a similar prognostic fashion, if enough noncancerous tumors had been available for comparison.

The great propensity of cancerous melanomas of skin and mucous membrane to involve local structures was indicated by the extension of 5 cancerous melanomas of the skin into contiguous tissues and by the recurrence (actually, continuing growth of residual tumor) of cancerous melanomas of mucous membrane in 7 dogs. The spread of cancerous melanomas from the skin to regional and distant lymph nodes and to lungs is relatively frequent, as observed in 12 dogs. The data for 3 cases of cancerous melanoma of mucous membrane which metastasized were meager by comparison, with regional lymph nodes affected in 2 dogs and the brain in 1. Examination of regional lymph nodes and brains in all dogs might have resulted in a greater incidence of metastases, except that necropsies made elsewhere usually omitted examination of them.

Only 1 dog with a primary cancerous melanoma of the skin was known to be alive and well at 7 months after operation, as compared with 15 dogs with noncancerous melanoma of the skin, of which 10 were alive and well at 3 to 44 months after excision and 5 died of other causes without recurrence at 7 to 32 months. Of 2 dogs with cancerous melanoma of mucous membrane, 1 had no recurrence at euthanasia, 15 months after surgery, and 1 was alive and well at 44 months. The 2 dogs with

noncancerous melanoma of mucous membrane were alive and well at 11 and 17 months.

The location of melanoblasts in epidermis, dermis, and hypodermis allowed a division of the noncancerous melanomas of the skin into 3 types[2]: dermal in 19, hypodermal in 6, and compound in 4. The 4 dogs with noncancerous melanoma of compound type were 6, 7, 8, and 10 years of age. No example of noncancerous melanoma of pure epidermal type was observed. The frequency of involvement of epidermis by melanoblasts in noncancerous melanoma in man diminishes[4] rapidly in the second decade and is rare after the age of 40 years. However, cancerous melanoma is often associated with anaplastic epidermal melanoblasts in man past this age.[2,4] In the analysis of the cases of cancerous melanoma with suitable material, it was found that in 6 of 24 tumors of the skin and 8 of 23 of mucous membrane the epidermis or the epithelium was infiltrated by benign and malignant melanoblasts. This important phenomenon could have been more frequent if surface ulceration had not been a common complication. The origin of at least 1 cancerous melanoma of the skin in a previously noncancerous hypodermal melanoma was suggested by the huge size of the primary tumor and the long clinical course (case 20, described before[6]).

The histopathologic features of noncancerous and cancerous melanoma are generally comparable in man and in the dog. No examples of so-called "juvenile melanoma,"[2,5] properly classified as an injured or altered noncancerous dermal melanoma, were found in this series of melanoma in dogs.

No special comment is necessary for the 2 new cases of cancerous melanoma of the eye, which has been well described.[9]

SUMMARY

Of 88 cases of melanoma in dogs, 59 primary tumors involved the skin; 29, mucous membrane; and 2, the eye. More than 50% of the melanomas of the skin and more than 90% of those of mucous membrane were cancerous. The male to female ratio for melanoma of the skin was 1.4 to 1.0; for melanoma of mucous membrane it was 3.1 to 1.0. Noncancerous melanoma of the skin was most frequent at 6 to 10 years of age; cancerous melanoma of skin and mucous membrane, at 11 to 15 years of age. Melanoma was most common in breeds with moderately or heavily pigmented skin, notably the Cocker Spaniel and the Scottish Terrier. Cancerous melanoma of mucous membrane tended to be of shorter duration than noncancerous or cancerous melanoma of skin. An approximate correlation between size and degree of aggressiveness was observed for melanoma of skin. Of 29 noncancerous melanomas of skin, 19 were of dermal type. Involvement of epidermis by malignant melanoblasts was recorded in 25% of cancerous melanomas of skin; involvement of epithelium, in 34% of those of mucous membrane. Two new cases of cancerous melanoma of the eye were reported.

Melanomas in man and in the dog were found to have the following similarities: age incidence for cancerous melanoma of skin and mucous membrane, frequency of noncancerous melanoma of the skin of head and trunk, types and frequency of noncancerous melanoma of the skin, frequent origin of cancerous melanoma of skin and mucous membrane in malignant melanoblasts of epidermis and epithelium, and histopathologic features of noncancerous and cancerous melanomas.

Melanomas in man and in the dog were found to have the following differences: higher frequency of noncancerous melanoma of the skin in man, lower incidence of cancerous melanoma of mucous membrane in man, equal sex incidence in man as compared to male sex dominance in the dog, frequent congenital appearance of noncancerous melanoma of skin in man as compared with the rarity in dogs under 5 years of age, high incidence of cancerous melanoma of lower extremities in man as compared to low frequency of involvement of posterior extremities in the dog, and more frequent occurrence of melanoma in persons with light skin as compared to high incidence in breeds of dogs with moderate to dark skin pigmentation.

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SUMMARIO IN INTERLINGUA

Tumores Melanoblastic in le Can

Inter 88 casos de melanoma in canes, 59 tumores primari interessava le pelle, 29 un membrana mucose, e 2 un oculo. Plus que 50% del melanomas del pelle e plus que 90% del melanomas de membrana mucose esseva cancerose. Le proportion masculo-feminin pro melanomas del pelle esseva 1,4 a 1,0, pro melanomas de membrana mucose 3,1 a 1,0. Non-cancerose melanoma del pelle esseve le plus frequente a etates de inter 6 e 10 annos. Cancerose melanoma de pelle e membrana mucose esseva le plus frequente a etates de inter 11 e 15 annos. Melanoma esseva le plus commun in racias con moderatemente o fortemente pigmentate pelles, notabilemente le espaniol cocker e le terrier scote. Melanoma cancerose de membrana mucose tendeva a durar minus longemente que melanoma non-cancerose de membrana mucose o que cancerose melanoma del pelle. Un correlation approximative esseva notate inter le dimensiones e le grado de aggressivitate pro melanomas del pelle. Inter 29 non-cancerose melanomas del pelle, 19 esseva del typo dermal. Affection del epiderme per maligne melanoblastos esseva constatate in 25% del cancerose melanomas del pelle. Affection de epithelio esseva presente in 34% del melanomas de membrana mucose. Duo nove casos de cancerose melanoma del oculo es reportate.

Esseva trovate que melanomas in le homine e in le can ha le sequente similitudes: Incidentia secundo le etate pro melanoma cancerose del pelle e de membrana mucose, frequentia de non-cancerose melanoma del pelle in capite e trunco, typos e frequentia de melanoma non-cancerose del pelle, le frequente origine de cancerose melanoma del pelle e de membranas mucose in maligne melanoblastos de epiderme e epithelio, e aspectos histopathologic de non-cancerose e cancerose melanomas.

Esseva trovate que melanomas in le homine e le can ha le sequente differentias: Un plus alte frequentia de non-cancerose melanoma del pelle in le homine, un plus basse incidentia de cancerose melanoma de membrana mucose in le homine, equal incidentia secundo le sexos in le homine per contrasto con le dominantia del sexo mascule in le can, le frequente occurrentia congenite de non-cancerose melanoma del pelle in le homine per contrasto con le raritate del condition in canes de etates de minus que 5 annos, le alte incidentia de melanoma cancerose in le extremitates inferior per contrasto con le basse frequentia de affection del extremitates posterior in le can, e le plus frequente occurrentia de melanoma in humanos con pelle blanc per contrasto con le alte incidentia in racias canin con moderate o forte pigmentation cutanee.

Studies on Immunity to *Uncinaria stenocephala* Infection in the Dog—Double Vaccination with Irradiated Larvae

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IT HAS BEEN shown that it is possible to produce a high degree of immunity to infection with the European hookworm, *Uncinaria stenocephala*, by the oral administration of irradiated infective larvae.[1] This technique was first applied to the bovine lungworm, *Dictyocaulus viviparus*, and it was found that the efficiency of the vaccine could be increased by giving 2 doses at an interval of approximately 4 to 6 weeks.[2] It was considered worthwhile to test the effect of administering 2 doses of irradiated 3rd-stage hookworm larvae to pups before subjecting them to challenge infection with normal larvae. At the same time, an opportunity was taken to examine the effect of a lower roentgen dose on the larvae.

MATERIALS AND METHODS

Twenty-two bitch pups of mixed breeding, 6 to 10 weeks of age, were purchased from a commercial source and were housed in wire-floored cages in strict isolation for 5 weeks before commencing the experiment. During this period, frequent parasitologic examinations were made of their feces to ensure that they were free of hookworm infection.

Feces containing hookworm eggs were obtained from experimentally infected dogs and were maintained in charcoal cultures for 12 days at room temperature before the infective larvae were used. The method of irradiation has been described previously.[2]

Fecal egg outputs in the experimental animals were estimated twice weekly by the McMaster method. The intestines were left for at least 1 hour after necropsy before being washed out. A complete count of all worms present in the bowel washings was then made.

Tissue taken for histologic examination was fixed in corrosive formol, dehydrated, cleared in an alcohol-amyl acetate-benzene series, and embedded in paraffin. Routine staining was by hematoxylin and eosin.

Six pups (group A) were each given 1,000 infective larvae subjected to 40,000 r, and another 6 (group B) were given 1,000 larvae irradiated with 20,000 r. These treatments were repeated 28 days later. It was intended that the immunity of all these animals should be challenged with normal larvae at a later date, but 1 animal in each group died of distemper during the experiment. Three pups (group C1) were given 1,000 normal larvae to test the infectivity of the larvae used to prepare the 1st batch of vaccine. These were killed after 28 days. Six pups (group C2) were given 1,000 normal larvae as a check on the culture used for the 2nd batch of vaccine. Three (C2-a) were destroyed 28 days later. The immunity of the other 3 (C2-b) was challenged on the 228th day, as was the immunity of the vaccinated animals. Three pups (C3) left uninfected until the 228th day were given 1,000 normal larvae as a control of the culture used to challenge the immunity of the vaccinated pups which had also been given 1,000 normal larvae. All were killed on the 250th day.

RESULTS

Control groups C1 and C2 had mean intestinal worm burdens of 630 ± 45 and 665 ± 70, respectively, indicating that the cultures used for the preparation of both batches of vaccine were highly infective. No direct attempt to assess the infectivity of the vaccine was made, but 1 animal from each group died of intercurrent disease 17 to 24 days after the 2nd dose; the pup from group A contained 196 hookworms and that from group B had 65. Further indirect indications of infectivity were the fecal egg counts on the 28th day, when the mean outputs of pups of groups A, B, and C1 were 8,500, 900, and 23,200 eggs per gram (e.p.g.), respectively (Fig. 1).

Revaccination produced no significant increase in egg production in either group, while the mean fecal egg outputs in pups of control group C2 rose to over 25,000 e.p.g. on the 56th day, *i.e.*, 28 days after infection. Eggs persisted in the feces of all animals in group A until the 210th day and in animals in group B until the 180th day. The vaccinated animals were given challenge doses on the 228th day and

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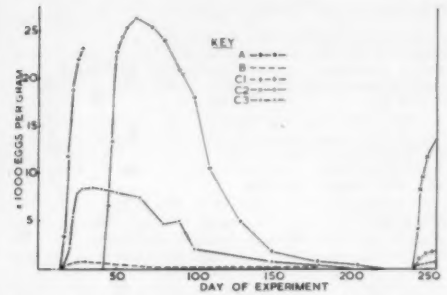

Fig. 1—Mean fecal egg counts for vaccinated and control pups.

were killed on the 250th day. During this period, the egg outputs of the dogs in group A rose to almost 500 e.p.g.; whereas, eggs were detected only in 1 animal in group B. The egg outputs in the corresponding control group C3 rose to 13,000 e.p.g. on the 250th day. The mean worm burdens at necropsy were 22.6 ± 14 in group A pups and 2.4 ± 3 in group B pups, as against 512 ± 34 in the control animals. This is a significant reduction, indicating that double vaccination with irradiated larvae had stimulated a high degree of immunity to reinfection. The final worm burden in the 3 animals of control group C2 which were submitted to challenge was significantly higher than that in either of the vaccinated groups (Table 1).

Pathologic changes.—There were no obvious intestinal lesions in the vaccinated animals following challenge, but in those infected with normal larvae, the feces were diarrheic and small focal hemorrhages were numerous, particularly in the upper half of the small intestine.

Histologic examination of the small intestines of animals given normal larvae revealed vascular dilatation and thrombosis associated with capillary necrosis and hemorrhage in the crypt region of the mucosa. Necrotic tracts extended down through the mucosa into the submucosa at some points. Vascular and necrotic lesions were absent in the vaccinated pups subjected to challenge, but small eosinophilic granulomas were prominent in the submucosa. Occasional foci of vascular change were present in the intestines of the 3 pups of control group C2 which were given challenge doses of normal larvae.

Discussion

These experiments verify the previous observations that irradiated larvae of *U. stenocephala* are capable of producing a high degree of resistance to reinfection.[1] A significantly greater number of worms was recovered from the pups given larvae irradiated with 20,000 r than from those given larvae irradiated with 40,000 r. The fecal egg output of pups in the former group was also higher, and it is obvious that a greater number of larvae reached maturity at this dose level. Comparison with the previous experiment, where the infectivity of the challenge larvae was similar, suggests that double vaccination produces a higher degree of resistance to reinfection than does single vaccination. In the present experiment, the roentgen dosages used were insufficient to produce attenuation of all the larvae; some reached maturity and laid viable eggs. If this method were to be applied in the field, a higher degree of partial inactivation would have to be obtained to minimize both the

TABLE 1—The Roentgen Levels Used in Immunizing Doses and the Immunogenicity of Irradiated Hookworm Larvae

Groups of dogs	No. in group	Treatment at day 1	Mean No. of worms at 28 days (e.p.g.)	Treatment at 28 days	Mean No. of worms at 56 days (e.p.g.)	Challenge at 228 days	Mean No. of worms at 245 days (e.p.g.)
Group A	5	1,000 larvae x 20,000 r	.....	1,000 larvae x 20,000 r	.....	1,000 normal larvae	22.6 ± 13.7
Group B	5	1,000 larvae x 40,000 r	.....	1,000 larvae x 40,000 r	.....	1,000 normal larvae	2.4 ± 2.9
Group C1	3	1,000 normal larvae	630 ± 45	.....	.....	.....	.....
Group C2a	3	.....	.....	1,000 normal larvae	665 ± 70	.....	.....
Group C2b	3	.....	.....	1,000 normal larvae	.....	1,000 normal larvae	90.0 ± 17.0
Group C3	3	.....	.....	.....	.....	1,000 normal larvae	512.0 ± 34.0

r = roentgens.

effect of the vaccine on the host and the potential of the latter as a vector of infection. This may be possible by using higher levels of irradiation.

SUMMARY

To artificially immunize pups against infection with *Uncinaria stenocephala*, a vaccine composed of irradiated infective larvae was used. A high degree of resistance to challenge followed treatment with 2 doses of this vaccine.

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SUMMARIO IN INTERLINGUA

Studios Relative al Immunitate Contra Infection per *Uncinaria stenocephala* in le Can. Duple Vaccination con Irradiate Larvas

Pro effectuar un immunitate artificial contra infection per *Uncinaria stenocephala* in canes infante, un vaccino esseva usate que consisteva de irradiate larvas infective. Un alte grado de resistentia contra le provocation infective sequeva le tractamento con duo doses del mentionate vaccino.

Spontaneous Abnormal Cardiac Arrhythmias and Conduction Disturbances in the Dog

A Clinical and Pathologic Study of 3,000 Dogs

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THE PREVALENCE of abnormal cardiac arrhythmias and conduction disturbances occurring spontaneously in dogs brought to a veterinary clinic has not been previously established. During an 18-month period, 3,000 dogs brought in consecutive sequence to the Small Animal Clinic of the University of Pennsylvania were screened for evidence of cardiovascular disease. There were 124 abnormal arrhythmias and conduction disturbances recorded in electrocardiograms taken from 95 of these dogs. Some arrhythmias and conduction disturbances were found during a screening examination; others appeared in subsequent electrocardiograms taken later in dogs selected for further studies.

LITERATURE REVIEW

The spontaneous occurrence of abnormal cardiac arrhythmias is a familiar concomitance of heart disease in dogs.[2, 15, 22] Several authors have reported electrocardiographic diagnoses of specific cardiac arrhythmias and conduction disturbances as follows: atrial premature beats[2, 14]; atrial fibrillation[2, 4, 6, 7, 11, 13, 14, 16, 18, 19, 20, 24, 32]; atrial flutter[6, 7, 13, 21]; ventricular premature beats[2, 4, 6, 14, 16, 22, 32]; atrioventricular nodal rhythm[13, 25]; paroxysmal ventricular tachycardia[4, 6, 13, 32]; sinus arrest[6]; sinuatrial block[6, 14, 16, 32]; atrioventricular block[2, 4, 6, 16, 32]; and bundle branch block.[2, 25]

In a series of 1,000 dogs screened for evidence of cardiovascular disease, the following cardiac arrhythmias were encountered: ventricular premature beats, paroxysmal ventricular tachycardia, incomplete atrioventricular block, complete atrioventricular block, bundle branch block, paroxysmal atrial tachycardia, and atrial fibrillation.[9]

The diagnosis and clinical significance of the more common arrhythmias and conduction disturbances in the dog have been discussed elsewhere.[5, 9]

MATERIALS AND METHODS

All dogs admitted to the Small Animal Clinic of the University of Pennsylvania during clinic hours were screened for evidence of cardiovascular disease. The brief screening examination consisted of auscultation of the 4 cardiac valve areas, palpation of the precordium and femoral arteries, and the recording of a single semiunipolar thoracic lead electrocardiogram.

Dogs in which evidence of cardiac abnormalities was found were studied further by means of complete 10-lead electrocardiograms, thoracic radiographs, and phonocardiograms. Special techniques such as cardiac catheterization, clinical laboratory procedures, blood pressure determination, and angiocardiography were used where applicable. Animals that died or were euthanatized were studied through gross and histologic postmortem examinations whenever possible.

An ink-writing* or heated-stylus electrocardiograph** was used for routine electrocardiograms. A photographic electrocardiograph† was employed for taking simultaneous electrocardiograms and phonocardiograms and for recording 2 electrocardiographic leads simultaneously. Complete electrocardiograms consisted of the bipolar limb leads I, II, III, augmented unipolar leads aVR, aVL, aVF and the unipolar precordial leads CV_6LL (CV_6L), CV_6LU (CV_6U), CV_5RL (CV_5) suggested by Lannek (Lannek's abbreviations for these leads are given in the parentheses).[26] In the precordial leads, the exploring electrode positions are at the sternal edge of the 6th left lower interchondral space (CV_6LL), at the level of the costochondral junction in the 6th left intercostal space (CV_6LU), and at the edge of the sternum in the 5th right interchondral space (CV_5RL). These precordial positions overlie the apex of the left ventricle, and the free walls of the left and right ventricles, respectively. In some cases, the unipolar lead V_{10} was taken also; the exploring electrode is placed over the spine of the 7th thoracic vertebra.

Neither sedatives nor anesthetics were given before electrocardiograms were taken. The animals were restrained in lateral recumbency with the right side down on a wooden table. Alligator clips placed on the lead wires were attached to the skin which had been moistened with electrode paste. Clipping or shaving of the hair was not necessary.

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From the School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

* Edin Electronic Cardiograph made by Edin Co., Worcester, Mass.

** Cambridge Versiscribe made by Cambridge Instrument Co., New York, N.Y.

† Sanborn Twin-Beam made by Sanborn Co., Cambridge, Mass.

Electrocardiographic abnormalities other than arrhythmias and conduction disturbances were recorded among the 3,000 dogs studied, but are not considered in this report.

In cases where arrhythmias or conduction disturbances were found, an attempt was made to determine the principal type of underlying cardiac disease by clinical methods (physical examination, radiography, clinical laboratory examination, cardiac catheterization, angiocardiography) and, whenever possible, by gross and histologic postmortem studies.

On the basis of clinical or necropsy findings, all dogs examined were placed in the following groups:

No Cardiac Disease.—In clinical studies, dogs in which the electrocardiographic change was the only detectable cardiac abnormality, and in which no condition known to produce cardiac disease was present (anemia, toxemia, electrolyte disturbances, generalized acute infection, *Dirofilaria immitis* infection), were placed in this category. Dogs in which cardiac lesions were absent on gross and histologic necropsy examinations were also included in this category.

Chronic Valvular Disease (Fibrosis of the Atrioventricular Valves).—Clinically, dogs over 4 years of age which had significant systolic murmurs [8] in the mitral or tricuspid valve areas, and in which signs of congenital heart disease, bacterial endocarditis, and anemia were absent, were placed in this category. In many cases, additional signs of chronic valvular disease were present (precordial thrills in the mitral or tricuspid areas, cardiac enlargement compatible with mitral or tricuspid insufficiency). Corresponding fibrosis of the mitral or tricuspid valve, or both, was confirmed in all dogs necropsied. Fibrosis of the mitral valve was observed most often. The tricuspid valve was frequently involved to a lesser degree, but isolated tricuspid valve fibrosis was rare. Fibrosis of the atrial endocardium and dilatation of the corresponding atrium were usually observed at necropsy in dogs in which mitral or tricuspid regurgitation was detected clinically.

Fibrosis of the atrioventricular valves was seen in nearly all of the dogs necropsied which were over 5 years of age. The degree of fibrosis varied from extreme thickening and deformation to slight thickening with little or no functional impairment of the valves. Murmurs or other signs of valvular regurgitation were not present in life in dogs with only slight thickening, and in these the atrioventricular valves did not appear insufficient when examined postmortem.

In this series, all except 3 of the dogs reported as having chronic valvular disease had murmurs. These dogs are included (Table 3) under the heading *Chronic Valvular Disease with Arteriosclerosis and Myocardial Disease,* since the microscopic appearance of the valves and myocardium was the same as the others in this group, but the valvular changes less severe.

Arteriosclerosis with Myocardial Disease.—In many of the dogs necropsied, focal myocardial necrosis and fibrosis accompanied arteriosclerotic lesions in the small intramyocardial coronary arteries. The arterial lesions were characterized by medial and intimal thickening often markedly narrowing the arterial lumen. Degenerative changes were evident at times in the thickened media, and thrombosis of small arteries was occasionally observed. Some degree of fibrosis of the atrioventricular valves almost invariably accompanied the arteriosclerosis and myocardial disease in the dogs studied postmortem.

Myocarditis.—Clinically, dogs with arrhythmias or conduction disturbances in the presence of signs of acute infection, but without signs of other cardiac disease, were placed in this category. This is a presumptive diagnosis, since noninflammatory lesions may produce similar electrocardiographic changes. On histologic examination, dogs with myocardial lesions predominantly inflammatory in nature were classified as having myocarditis.

Congenital Heart Disease.—In all dogs placed in this category, congenital lesions were confirmed at surgery or necropsy. Because of the small number of congenital cases included in this study, no correlation on the basis of specific cardiac anomalies was attempted.

Miscellaneous.—This category includes a variety of less common cardiac lesions, such as aortic body tumors, metastatic neoplasms of the heart, cor pulmonale due to *Dirofilaria immitis* infection, and other conditions such as anemia, toxemia, and severe electrolyte disturbances, all known to cause secondary myocardial changes that may result in electrocardiographic abnormalities.

Digitalis Toxicosis.—In a few dogs in this series, arrhythmias or conduction disturbances appeared after treatment with digitalis glycosides. In most instances, they were thought to be evidence of digitalis toxicosis, and as such are considered separately in this report. Although it might be argued that disturbances in conduction and rhythm which occur under the influence of digitalis are not spontaneous, they are included here for the sake of completeness. Further, it is not possible to say that digitalis was wholly responsible for the abnormalities observed, since advanced cardiac disease was present in each dog given digitalis.

Instances of incomplete atrioventricular block which followed digitalis therapy are not included in this study, since prolongation of atrioventricular conduction occurs commonly in the dog at therapeutic levels of digitalis therapy, and is not usually considered a manifestation of digitalis toxicosis.

RESULTS

Among 3,000 dogs screened, 124 examples of 15 different arrhythmias and conduction disturbances were recorded in 95 dogs. Table 1 presents the over-all frequency of occurrence of the various abnormalities. A number of dogs had more than one abnormality concurrently, or developed additional ones during the time they were being

TABLE 1—Types and Frequency of Occurrence of Spontaneous Abnormal Arrhythmias and Conduction Disturbances in 95 Dogs from a Sample of 3,000 Dogs

Ventricular premature beats	43
Atrial premature beats	14
Atrial fibrillation	13
Incomplete atrioventricular block	12
Incomplete atrioventricular block with dropped beats	12
Paroxysmal ventricular tachycardia	8
Ventricular parasystole	4
Paroxysmal atrial tachycardia	3
Atrioventricular nodal premature beats	3
Atrioventricular dissociation	3
Atrial flutter	2
Complete heart block	2
Left bundle branch block	2
Right bundle branch block	2
Wolff-Parkinson-White syndrome	1
Total	124

studied. The ages of dogs with arrhythmias and conduction disturbances ranged from 6 months to 19 years. The mean age was 7.9 years. There were 58 males and 37 females.

Clinical Studies.—Table 2 relates the types of arrhythmias and conduction disturbances to the diagnoses in 58 dogs studied clinically but not necropsied. Table 4 includes 3 additional dogs which were studied clinically but in which the electrocardiographic change was attributed to digitalis toxicity. In Table 2, the electrocardiographic abnormalities were associated with signs of chronic valvular disease in 29 of 70 instances. In 16 the diagnosis was myocarditis. Miscellaneous conditions, such as heartworm infection, anemia, and severe electrolyte disturbances, were present in 10 instances. The only dog with congenital heart disease included in Table 2 had a patent ductus arteriosus. Ventricular premature beats disappeared after corrective surgery.

In 14 instances, cardiac arrhythmias or conduction disturbances occurred in the absence of other clinical signs of cardiac disease, signs of systemic infection, or conditions potentially injurious to the myocardium.

Postmortem Studies.—Table 3 relates the arrhythmias and conduction disturbances to the types of pathologic changes found in the heart in gross and histologic postmortem studies of 28 dogs. An additional 6 dogs which were necropsied had signs of digitalis toxicosis at sometime prior to death or euthanasia and are considered separately in Table 4.

Chronic Valvular Disease, Coronary Arteriosclerosis, and Myocardial Disease.—Of the 44 arrhythmias and conduction disturbances observed (Table 3), 28 occurred in 20 older dogs with concomitant fibrosis of the atrioventricular valves, arteriosclerosis of the small intramyocardial coronary arteries, and disseminated focal myocardial necrosis and fibrosis. The only dog necropsied in which arterial thickening and areas

TABLE 2—Relationships Between Spontaneous Abnormal Arrhythmias and Conduction Disturbances and Various Types of Cardiac Disease Diagnosed Clinically in 58 Dogs

EKG change	No other signs of cardiac disease	Chronic valvular disease	Myocarditis	Congenital heart disease	Miscellaneous	Total
Ventricular premature beats	5	8	9	1	3	26
Atrial premature beats	..	10	..	..	..	10
Incomplete atrioventricular block	4	4	1	..	2	11
Incomplete atrioventricular block with dropped beats	2	3	2	..	3	10
Paroxysmal ventricular tachycardia	..	..	3	..	1	4
Ventricular parasystole	1	1	..	..	..	2
Paroxysmal atrial tachycardia	..	1	..	..	..	1
Complete heart block	..	1	..	..	1	2
Atrioventricular nodal premature beats	..	1	1	..	..	2
Right bundle branch block	1	..	..	..	..	1
Wolff-Parkinson-White syndrome	1	..	..	..	..	1
Total	14	29	16	1	10	70

TABLE 3—Relationships Between Spontaneous Abnormal Arrhythmias and Conduction Disturbances and Cardiac Lesions in 28 Dogs Necropsied

EKG change	Cardiac lesion					
	No cardiac disease	Chronic valvular disease with arteriosclerosis and myocardial disease	Myocarditis	Congenital heart disease	Miscellaneous	Total
Ventricular premature beats	..	7	2**	1†	3	13
Atrial premature beats	..	2	..	..	2	4
Atrial fibrillation	..	12	..	1	..	13
Incomplete AV block*	..	..	..	..	1	1
Incomplete AV block with dropped beats*	1	..	..	..	1	2
Paroxysmal ventricular tachycardia	..	2	2**	..	..	4
AV nodal premature beats*	..	..	.	1	..	1
Paroxysmal atrial tachycardia	..	..	..	..	1	1
Atrial flutter	..	2	..	..	..	2
Left bundle branch block	..	2	..	..	..	2
Right bundle branch block	..	1	..	..	..	1
Total	1	28	4	3	8	44

* AV = atrioventricular. ** One dog had acute myocarditis superimposed on chronic valvular disease, arteriosclerosis, and myocardial disease. † Arteriosclerosis with areas of myocardial necrosis and fibrosis were also observed in this dog.

of myocardial necrosis were not associated with chronic valvular disease had congenital aortic stenosis. In this dog, the atrioventricular valves appeared normal.

The foci of myocardial disease were most common in the left ventricle and appeared to be microscopic areas of ischemic necrosis in various stages of resorption and repair (Fig. 7). The arterial lesions were characterized by medial and intimal thickening. The internal elastic membrane was frequently split and fragmented. In advanced cases, degenerative changes were often superimposed on these arterial lesions, resulting in loss of nuclei and replacement of large portions of the arterial wall by a homogeneous eosinophilic hyaline material.

Subendothelial cushions were frequently observed in the small arteries of this group. These structures, which on cross-section are broad at the base, and taper to a rounded, blunt tip, sometimes almost completely filled the arterial lumen. They appeared to be composed of smooth muscle and elastic tissue arising from the arterial wall. Such subendothelial cushions have been observed in apparently normal hearts.[5] In our cases, when degenerative changes of the arterial wall occurred, similar changes were usually also present in the arterial cushions.

Advanced fibrosis of the atrioventricular valves was frequently accompanied by dilated atria with areas of endocardial fibrosis. These changes were presumably due to the effects of increased intra-atrial pressure resulting from atrioventricular valve insufficiency. The mitral valve was most often involved, and these changes usually were most severe in the left atrium. Microscopically, areas of myocardial necrosis, infiltration with lymphocytes and mononuclear phagocytes, edema, and fibrosis were scattered throughout the atrial myocardium. The myocardial fibers appeared thin and curled (Fig. 8).

Myocarditis.—Myocardial lesions which

TABLE 4—Arrhythmias and Conduction Disturbances Which Occurred Following Digitalis Therapy in 9 Dogs with Congestive Heart Failure

EKG change	Underlying cardiac disease	No.
Ventricular premature beat	Chronic valvular disease* arteriosclerosis and myocardial disease	2
	Chronic valvular disease	2
Ventricular parasystole	Chronic valvular disease	1
	undiagdosed myocardial disease	1
Atrioventricular dissociation	Chronic valvular disease* arteriosclerosis and myocardial disease	1
	Patent ductus arteriosus*	1
	cor pulmonale due to heartworms*	1
Paroxysmal atrial tachycardia	Congenital pulmonic stenosis*	1
	Total	10**

* Postmortem diagnosis. ** One dog had ventricular parasystole as well as ventricular premature beats following digitalis therapy.

TABLE 5—Relationships Between Types of Abnormal Arrhythmias and Conduction Disturbances and the Types of Heart Disease Observed in 3,000 Dogs*

Diagnosis	Arrhythmia or conduction disturbance				
	Atrial	Junctional or ventricular	Atrial and junctional or ventricular	None	Total
Chronic valvular disease	16/210(8%)	23/210 (11%)	6/210(3%)	165/210 (78%)	210
Other types of heart disease	3/80 (4%)	26/80 (33%)	0	51/80 (63%)	80
No other evidence of heart disease	0	(15/2,710 (1%)	0	2,695/2,710(99%)	2,710
Total	19	64	6	2,911	3,000

* The numbers in this table represent dogs rather than instances of occurrence of the arrhythmia or conduction disturbance as in other tables. Six dogs in which the only electrocardiographic charge was thought to be due to digitalis toxicosis are included under column 5 ("none").

were predominantly inflammatory occurred in 2 of the dogs necropsied. In one case, focal, purulent myocarditis involving the ventricles and atria was superimposed on chronic valvular disease and arteriosclerotic lesions of the small coronary arteries. In another, there was diffuse infiltration of the interstitial tissue with inflammatory cells. In both of these animals there were ventricular premature beats and paroxysmal ventricular tachycardia in life.

Miscellaneous.—Arrhythmias and conduction disturbances occurred 8 times in this group, including 2 dogs with malignant aortic body tumors, 2 with metastatic tumors involving the heart, 1 with myocardial degeneration associated with severe anemia, and 1 with a parasitic granuloma of the myocardium.

Congenital Heart Disease.—Arrhythmias and conduction disturbances occurred 3 times in association with congenital heart disease. This group included 1 dog with aortic stenosis, and 2 with congenital pulmonic stenosis (1 with patent foramen ovale as well). Either right or left ventricular hypertrophy was present in each of these cases. The small coronary arteries were thickened and areas of myocardial necrosis were seen in the dog with aortic stenosis. Emboli were observed in the small coronary arteries of the dog with pulmonic stenosis and patent foramen ovale.

No Heart Disease.—Only one of the dogs necropsied in which arrhythmias or conduction disturbances were observed had no detectable pathologic changes in the heart. This animal had clinical and postmortem signs of distemper with encephalitis. Incomplete AV block had been observed during life.

Clinical and Pathologic Correlations in Specific Arrhythmias—General.—Early in the study it appeared that arrythmias and conduction disturbances arising from ectopic centers of impulse formation in the atrium (atrial premature beats, paroxysmal atrial tachycardia, atrial flutter, atrial fibrillation) were almost always associated with insufficiency of the mitral valve resulting from chronic valvular disease. This observation is borne out in Table 5. Of 210 dogs with chronic valvular disease, 16 (8%) had atrial arrhythmias alone, and 6 (3%) had atrial arrhythmias with junctional or ventricular arrhythmias and conduction disturbances. Of 80 dogs with other types of heart disease, only 3 (4%) had atrial arrhythmias.

Arrhythmias and conduction disturbances of junctional or ventricular origin were often associated with other types of heart disease. Of 80 dogs with other types of heart disease, 26 (33%) had arrhythmias and conduction disturbances of junctional or ventricular origin. Of the 210 dogs with chronic valvular disease, 23 (11%) had junctional or ventricular arrhythmias and conduction disturbances, and 6 (3%) had atrial arrhythmias as well.

In the sample considered in this study, the actual numbers of dogs having arrhythmias and conduction disturbances of junctional or ventricular origin are distributed almost equally between chronic valvular disease and other types of heart disease. This appears to be due to the relatively large number of dogs with chronic valvular disease.

In this series, atrial arrhythmias did not occur without other evidence of cardiac disease, while arrhythmias and conduction disturbances of junctional or ventricular origin occurred 15 times without other signs

of heart disease. In only 1 of the 15 was a necropsy performed and the absence of gross or microscopic lesions of the heart confirmed.

Of the 95 dogs with arrhythmias and conduction disturbances, 30 had unmistakable clinical or postmortem signs of congestive heart failure. Of these, 14 had atrial arrhythmias, 10 had junctional or ventricular arrhythmias and conduction disturbances, and 6 had both.

In 9 dogs treated for congestive heart failure, 10 arrhythmias and conduction disturbances followed digitalis therapy (Table 4). Ventricular premature beats occurred 4 times, atrioventricular dissociation 3 times, and ventricular parasystole twice. Atrial fibrillation was temporarily converted to paroxysmal atrial tachycardia with block following treatment with intravenous ouabain in 1 dog. The 1 dog, in which 2 abnormalities occurred, had both ventricular parasystole and coupled ventricular premature beats.

Ventricular Premature Systoles and Paroxysmal Ventricular Tachycardia.—Ventricular premature beats were the most frequently recorded abnormality. Usually they were unifocal in origin and were coupled to the previous normal beat by a constant interval (Fig. 1A). Multifocal ventricular premature beats occurred in a smaller number of dogs (Fig. 1B). Paroxysms of ventricular tachycardia were observed in 8 dogs which also had unifocal or multifocal ventricular premature beats (Fig. 1C). Clinically, ventricular premature beats were associated with a variety of types of cardiac disease. In 5 instances, they were the only clinically detectable cardiac abnormality and occurred without evidence of any other exciting cause. In 4 instances, ventricular premature beats were attributed to digitalis toxicosis (Table 4).

The hearts of 15 dogs in which ventricular premature beats were consistently present were studied histologically. Paroxysms of ventricular tachycardia occurred in 4 of these. Pathologic changes were present in the ventricular myocardium of each. Nine were old dogs which had arteriosclerosis of the small coronary arteries and foci of myocardial necrosis and fibrosis throughout the ventricles. In 2 of these 9, ventricular premature beats had occurred following digitalis therapy (Table 4). Fibrosis of the atrioventricular valves was present in each of these hearts. Two old dogs with ventricular premature beats and paroxysmal ventricular tachycardia had widespread myocarditis.

An 8-month-old dog with congenital subaortic stenosis had frequent ventricular premature beats (Fig. 1A). On histologic examination, there were numerous areas of myocardial necrosis and fibrosis in the ventricles, accompanied by markedly thickened intramyocardial coronary arteries. The left ventricle was hypertrophied. The atrioventricular valves appeared normal.

Three of the necropsied dogs with ventricular premature beats are included in the *Miscellaneous* category in Table 3. Metastatic tumors involved the myocardium in 2, while the 3rd had cor pulmonale associated with heartworm infection.

Atrial Premature Beats, Paroxysmal Atrial Tachycardia, and Atrial Flutter.—Atrial premature beats (Fig. 2A) were the most frequently observed atrial arrhythmia. They were accompanied by signs of chronic valvular disease in most instances. In 8 of 14 dogs, congestive heart failure was present.

Paroxysmal atrial tachycardia (Fig. 2B) was recorded 3 times in this series. In 1 instance, the arrhythmia preceded atrial fibrillation in a dog with mitral valve insufficiency due to chronic valvular disease. In another dog, a malignant aortic body tumor infiltrated the atrium (Fig. 2B). The 3rd dog had congenital pulmonic stenosis; atrial fibrillation was temporarily converted to paroxysmal atrial tachycardia with block following digitalis therapy (Table 4). Both the dog with chronic valvular disease and the one with congenital pulmonic stenosis had signs of congestive heart failure.

Atrial flutter was recorded twice. Both of these dogs had severe mitral valve insufficiency due to chronic valvular disease, and each had clinical and postmortem signs of "left heart failure." One developed atrial fibrillation terminally.

Atrial Fibrillation.—Atrial fibrillation was observed in 13 dogs, all of which were necropsied. Advanced fibrosis of the mitral valve was found in 12. The tricuspid valve was involved to a lesser degree in most. Each of the 12 had mitral insufficiency, an extremely dilated left atrium, and clinical and postmortem signs of "left-sided" congestive heart failure. Arteriosclerotic changes of the small coronary arteries and foci of myocardial necrosis were present in

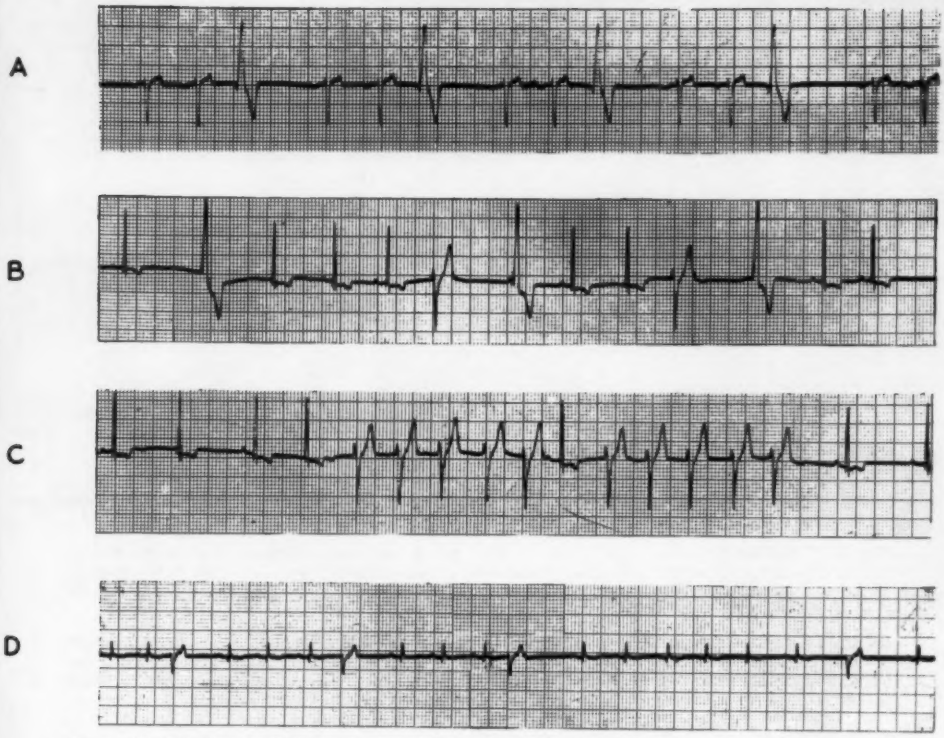

Fig. 1—Ventricular Arrhythmias. (A) Lead aVR. Coupled unifocal ventricular premature beats occurring every 3rd beat in an 8-month-old Newfoundland with congenital aortic stenosis. At necropsy, the left ventricle was markedly hypertrophied. Microscopic examination revealed thickening of the small coronary arteries and widespread focal myocardial necrosis throughout the left ventricle. The upright direction of the premature QRS complexes in this lead suggests that they originate in the left ventricle.

B) Lead II. Twice in midrecord, coupled ventricular premature beats with chiefly inverted QRS complexes are followed by ectopic ventricular beats from another focus (upright QRS complexes). These 2nd ectopic beats also are coupled to the 1st by a constant interval, but occur after a long pause. An ectopic beat of the same configuration follows a normal beat by the same long coupling interval at the beginning of the record. Because of the long interval between these ectopic beats and the preceding beat, they might be considered as ventricular escape beats with fixed coupling. However, this terminology has not been widely adopted and the matter remains controversial.

These abnormalities occurred in a 10-year-old female part Collie with acute myocarditis.

C) Lead II. Paroxysmal ventricular tachycardia. This record and that in B are from the same dog. Two paroxysms of ventricular tachycardia with QRS complexes of the same configuration as one of the types of coupled ventricular premature beats in B occur in midrecord. The 2 paroxysms are separated by a conducted sinus beat with a prolonged P-R interval. The first beats of the 2 runs of ventricular tachycardia are coupled to the previous normal beats by the same interval as the similar ventricular premature beats in B.

D) Lead I. Ventricular parasystole in an 11-year-old mongrel with no other evidence of cardiac abnormality. Notice that the bizarre ectopic QRS complexes are not coupled to the previous normal beats by a constant interval. The interval between ectopic beats 1 and 2, and 2 and 3 is 1.82 seconds, while the interval between 3 and 4 is 3.64 seconds, twice the smaller intervals. This suggests an ectopic ventricular pacemaker which discharges at a constant rate independent of the normal, dominant rate. The ectopic pacemaker becomes evident only when it discharges at a time when the ventricle is not in the refractory period of a normal beat.

the ventricles of each. Ventricular premature beats also occurred in 3 of these 13 dogs.

The 13th dog with atrial fibrillation had signs of "right-sided" congestive heart failure and, at necropsy, was found to have congenital pulmonic stenosis with hypertrophy and dilatation of the right ventricle and marked dilatation of the right atrium.

Incomplete Atrioventricular Block and Incomplete Atrioventricular Block with Dropped Beats.—Incomplete atrioventricular block occurred spontaneously in 12 dogs as evidenced by prolongation of the P–R interval beyond 0.15 second. Clinically, 7 of these dogs had other evidence of

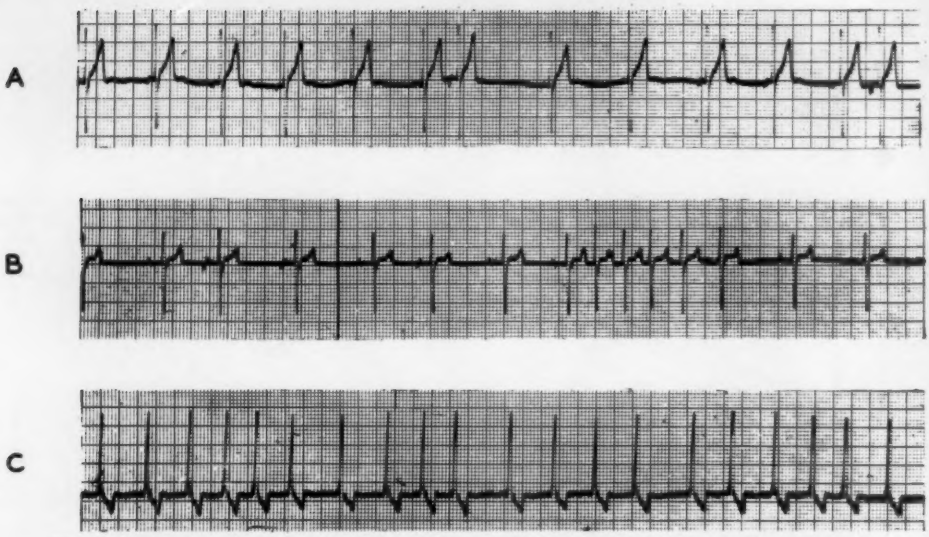

Fig. 2—Atrial Arrhythmias.—(A) Lead CV_5RL. Atrial premature beats in an 11-year-old male Cocker Spaniel with mitral insufficiency and left atrial dilatation due to mitral valve fibrosis. The P-waves are normally inverted or diphasic in this lead. Notice that the P-waves of the premature beats are more deeply inverted than the others.

B) Lead CV_5RL. Paroxysmal atrial tachycardia in a 9-year-old male Boxer with an aortic body tumor which infiltrated the right atrium. The P-waves vary in shape from beat to beat, and the P-R interval is slightly prolonged (0.16 sec.). A run of atrial tachycardia occurs in which the atrial and ventricular rates triple. During the run of atrial tachycardia the P-waves are partially buried in the preceding T-waves.

C) Lead CV_6LL. Atrial fibrillation in a 13-year-old male Doberman Pinscher with chronic valvular disease and congestive heart failure. Notice the absence of P-waves and the presence of irregular undulations of the base line (fibrillation waves). The ventricular rate is approximately 140 per minute. Postmortem examination of the heart revealed thickening and insufficiency of the mitral valve, and marked dilatation of the left atrium. Areas of myocardial necrosis were observed throughout the left atrium on microscopic examination (Fig. 8).

cardiac disease, but 4 did not. The only dog necropsied had widespread myocardial degeneration occurring in association with a severe anemia.

Incomplete atrioventricular block with dropped beats was observed in 12 dogs. Clinically, 8 of these had other signs of heart disease. Two dogs with this disturbance were necropsied. One had a parasitic granuloma in the region of the atrioventricular node; the 2nd had lesions of canine distemper elsewhere in the body but, microscopically, no detectable pathologic change in the heart.

Incomplete atrioventricular block was observed in a number of dogs following digitalis therapy, but these were not included in this study.

Complete Atrioventricular Block.—Complete atrioventricular block was recorded twice. In 1 dog, primary adrenocortical insufficiency was present. During adrenal crisis in which serum potassium was elevated and sodium markedly decreased, this conduction disturbance appeared.[20] Within hours following cortisone therapy and intravenous infusion of sodium chloride, the electrocardiogram returned to normal. No subsequent evidence of cardiac disease has been evident in this dog.

In the 2nd dog, signs of chronic valvular disease and congestive heart failure were present, but necropsy was not possible. Another dog with complete atrioventricular block, not included in this study, had an aortic body tumor which extensively infiltrated the right atrium, extending as far as the tricuspid valve (Fig. 3B).

Ventricular Parasystole.—Ventricular parasystole (Fig. 1D) was recorded in electrocardiograms from 4 dogs. None of these were necropsied. One dog had clinical evidence of "left-sided" heart failure due to myocardial disease of undetermined type. This dog had multifocal ventricular premature beats before digitalis therapy, but developed ventricular parasystole coincident with other signs of digitalis toxicosis (Table 4). In a 2nd dog with chronic valvular disease and congestive heart failure, ventricular parasystole occurred coincident with signs of digitalis toxicosis and disap-

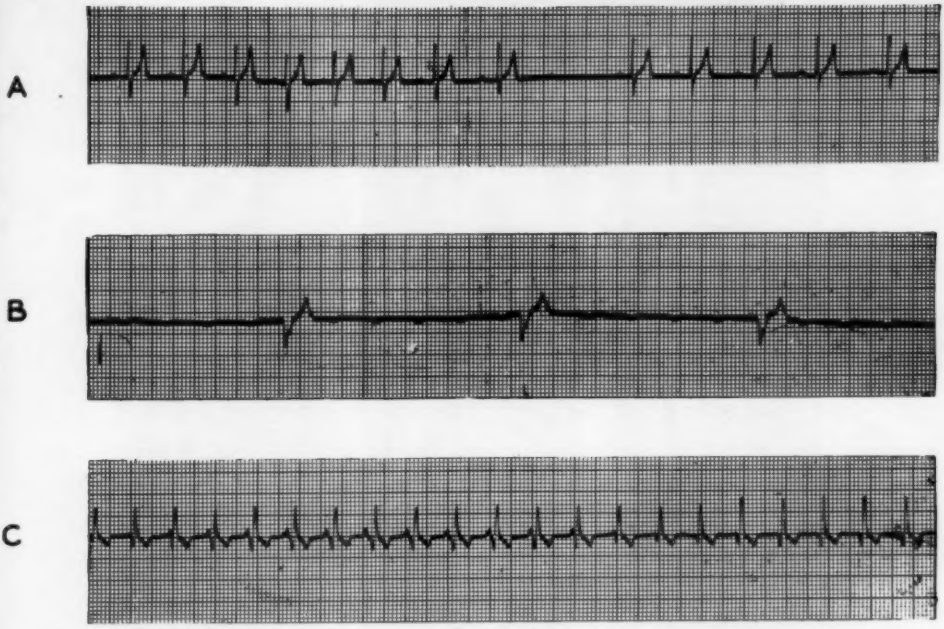

Fig. 3—Atrioventricular Conduction Disturbances. (A) Lead CV_5RL. Incomplete atrioventricular block with dropped beats showing the Wenckebach phenomenon in a 9-year-old female Miniature Poodle with no other evidence of cardiac abnormality. The P-R intervals become progressively longer (0.12 to 0.16 sec.) from left to right until a ventricular complex fails to occur in midrecord. The subsequent P-R interval is shortened to 0.12 second.

B) Lead II. Complete atrioventricular block with idioventricular rhythm in a 4-year-old male Boxer dog which had a malignant aortic body tumor infiltrating the atrium. The P-waves are inverted (normally upright in this lead) suggesting an ectopic atrial pacemaker. The atrial rate is 150 and the P-waves are not consistently related to the QRS complexes. The ventricular rate is 30 to 40 and the QRS complexes are bizarre, indicating an ectopic ventricular pacemaker.

C) Lead CV_5LU. Atrioventricular dissociation due to digitalis toxicity in a 6-year-old male Labrador Retriever with congestive heart failure. The animal had cor pulmonale due to Dirofilaria immitis infection. The P-waves migrate progressively into the QRS complexes until they disappear completely. Notice that the Q-wave becomes smaller, and the R-wave higher in amplitude where the P-waves become completely buried in the QRS complexes.

peared following withdrawal of the drug. Another dog had clinical signs of chronic valvular disease, while the 4th had no other clinically detectable cardiac abnormality.

Atrioventricular Dissociation.—Atrioventricular dissociation occurred 3 times in association with various types of cardiac disease. In each instance the animal was under treatment with digitalis at the time the abnormality appeared, and in each it was considered a manifestation of digitalis toxicosis (Fig. 3C, Table 4).

Atrioventricular Nodal Premature Beats. Atrioventricular nodal premature beats were observed in 3 dogs. They were infrequent and accompanied other evidence of cardiac disease. The 1 dog necropsied had congenital pulmonic stenosis and a patent foramen ovale with a venous-arterial shunt. Polycythemia was present and arterial thromboses were observed in the small coronary arteries and throughout the body.

Bundle Branch Block.—Tracings resembling experimental right and left bundle branch block[10,27-29,31] were observed in this series (Fig. 4, 5). Two dogs with a left bundle branch block pattern had marked arteriosclerosis of small coronary arteries, areas of necrosis throughout the ventricle, and fibrosis of the atrioventricular valves. One of the dogs with right bundle branch block pattern was young, and had no clinical signs of cardiac disease. The other had extensive right ventricular dilatation and congestive heart failure associated with fibrosis and insufficiency of the mitral and tricuspid valves. When the latter dog was necropsied, the pericardial sac was identified as a vestigial fringe of tissue at the base of the heart. Whether the lack of a pericardial sac was congenital in this case, or whether the sac had been ruptured due to trauma at some previous time, could not be determined. Microscopic examination of the

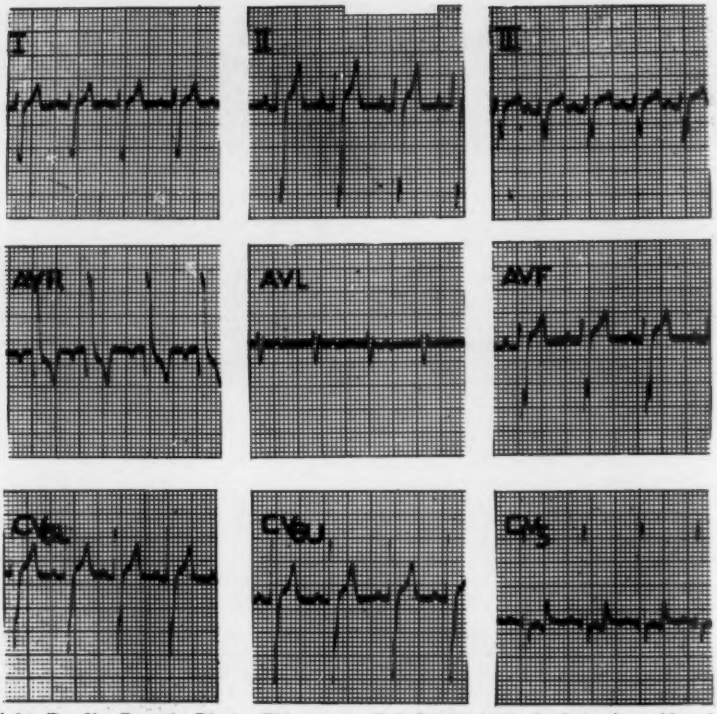

Fig. 4—Right Bundle Branch Block. This conduction disturbance occurred in a 2-year-old Airedale with no other evidence of cardiovascular disease. The QRS interval is prolonged (0.08 sec.). The major (widest) QRS deflection is negative in Leads I, II, III, aVL, aVF, CV_6L, and CV_6U, and positive in aVR and CV_5, indicating delayed right ventricular depolarization. This pattern is characteristic of that found in experimental bundle branch block. [20, 27, 28, 29, 31] The prominent initial R deflection in the leads having widened, notched S-waves, is attributed by Wilson and associates to an early activation of certain portions of the left ventricle.[30] In man, right bundle branch block patterns are usually characterized by negative major QRS deflections in Leads I and II, and positive major QRS deflections in Lead III. Patterns resembling those experimentally produced in the dog may occur in persons having a vertical heart position.[37]

ventricular myocardium revealed thickening of small coronary arteries, myocardial necrosis, and fibrosis.

Wolff-Parkinson-White Syndrome.—Electrocardiographic changes indicative of accelerated atrioventricular conduction (Fig. 6) occurred in a Wire-Haired Fox Terrier pup brought to the clinic for the treatment of demodectic mange. Thorough examination revealed no other cardiac abnormality.

Discussion

The occurrence of abnormal arrhythmias and conduction disturbances in 95 of 3,000 dogs examined consecutively as they were presented to a veterinary clinic indicates that such abnormalities are not rare. Clinical and postmortem studies supported the view that they are most often associated with acquired heart disease in older dogs. That myocardial lesions were demonstrated histologically in 33 of 34 necropsied dogs with abnormal arrhythmias or conduction disturbances, supports the obvious premise that these electrocardiographic abnormalities, when they occur spontaneously in the dog, usually result from pathologic changes in the myocardium or conduction tissue of the heart.

Abnormal arrhythmias and conduction disturbances in themselves are not generally believed to indicate the cause or nature of myocardial disease, but may reflect the location or severity of the change. When considered in the light of other signs, however, their presence may strongly suggest a specific type of myocardial disease (e.g., in man, the occurrence of incomplete atrioventricular block in the presence of signs of rheumatic fever suggests active rheumatic carditis).

A number of associations appear to indicate a relationship between given arrhythmias and conduction disturbances and certain types of cardiac disease. The authors are aware of the hazards of drawing definite conclusions from clinical and postmortem hospital data, because of the diffi-

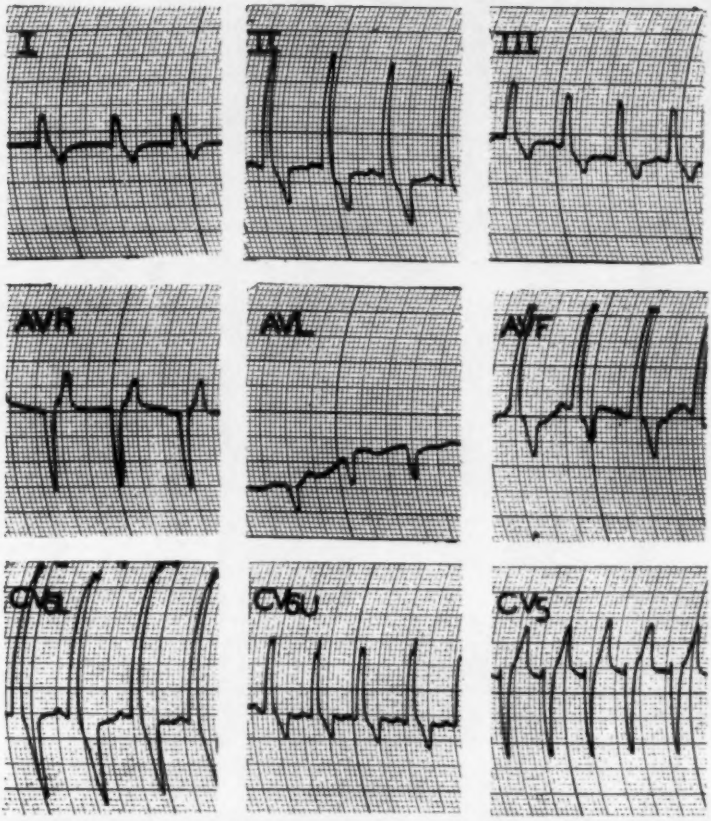

Fig. 5—Left Bundle Branch Block. This conduction disturbance occurred in an 11-year-old English Bulldog. In addition to aortic and mitral valve fibrosis, numerous foci of myocardial necrosis throughout the ventricles were observed at necropsy. Histopathologic examination of the heart revealed medial thickening, hyalinosis, and thrombosis of small coronary arteries with ischemic necrosis of small areas of myocardium. The delayed conduction presumably resulted from the effect of extensive myocardial disease on the left bundle branch or its main subdivisions. The major portion of the QRS complex is prolonged (exceeds 0.07 sec.) and is upright in all 3 standard limb leads, aVF, and leads over the left precordium, while it is inverted in aVR, aVL, and CV_5. This pattern is characteristic of experimental left bundle branch block in dogs (section of the left bundle branch).[20,27,28,31] A small Q-wave is present in Lead I and a small R-wave in CV_5. Although initial QRS forces in left bundle branch block are classically in a right to left rather than the normal left to right direction, such a small Q_1 is often present in experimental left bundle branch block in dogs.[31]

In man, if initial QRS forces are markedly rightward in direction prior to the development of left bundle branch block, the Q-waves in Lead I, and R-waves in the precordial lead corresponding to CV_5RL may not disappear, but merely be shortened in duration and smaller in amplitude.[12]

In left bundle branch block in man, the major deflection of the QRS complex is usually upright in Lead I and inverted in Lead III (discordant), although in individuals with a vertical heart position, the QRS may be upright in Leads I, II, and III, as in the dog.[17]

culty of assessing the biases operating in a sample not representative of the general population. Statistical tests applied to certain of the data indicate that some of the associations are highly significant. The results of the tests are not presented, however, to avoid conveying a false sense of validity.

The association between atrial arrhythmias and chronic valvular disease is not unexpected. Dilatation of the left atrium is a frequent consequence of the mitral insufficiency which characteristically results from chronic valvular disease. Increased intra-atrial pressure resulting from the regurgitation of blood into the atrium during ventricular systole causes stretching of the atrial myocardial fibers; associated with this is necrosis, interstitial edema, and cellular infiltration (Fig. 8). These changes constitute severe myocardial disease which might be expected to give rise to ectopic centers of impulse formation.

Arrhythmias and conduction disturbances of junctional or ventricular origin were

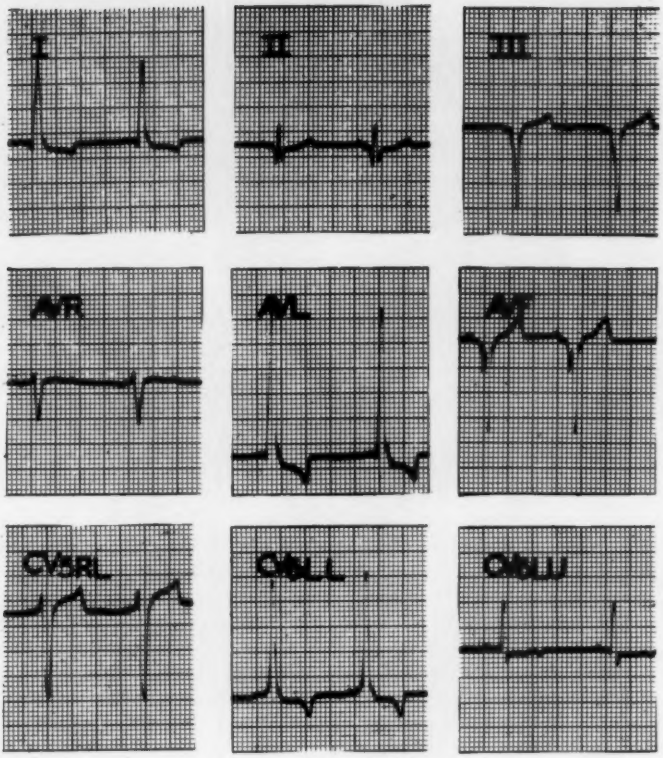

Fig. 6—Wolff-Parkinson-White Syndrome. This record was taken at 50 mm. per second, twice the conventional speed of clinical electrocardiograms. Notice the short P-R interval (0.05 sec. in Lead II), the prolonged QRS interval (0.08 sec.), and the slurred upstroke of R in CV_6LL (delta wave). The mean QRS axis is markedly leftward in direction (approx. −30 degrees), suggesting that aberrant atrioventricular conduction pathways initiate ventricular depolarization in the right ventricle. This conduction disturbance occurred in a 2-month-old male Wire-Haired Fox Terrier with no other detectable cardiac abnormality.

found with a variety of types of heart disease, including chronic valvular disease, myocarditis, congenital heart disease, metastatic neoplasms of the heart and cor pulmonale due to heartworm infection. In the dogs necropsied, the most common lesions of the ventricular myocardium were focal myocardial necrosis and fibrosis accompanying arteriosclerosis of the small intramyocardial coronary arteries. Most of the ventricular arrhythmias and conduction disturbances in the necropsied group appear to be caused by these changes. The consistent concurrence of chronic valvular disease in these dogs is striking. At the present time, it is not clear whether chronic valvular disease and arteriosclerosis of the small coronary arteries with focal myocardial necrosis and fibrosis have a common cause, have different causes, or are related as cause and effect, but there can be no doubt that they are very commonly found together in the hearts of aging dogs.

A number of arrhythmias and conduction disturbances were attributed to myocarditis in the dogs examined clinically only, but a primarily inflammatory process was present in the hearts of only 2 of the dogs necropsied (4 arrhythmias). Transient myocarditis probably accompanies a variety of infections in dogs.

Arrhythmias or conduction disturbances occurred 15 times without other clinical signs suggesting cardiac disease in the group of dogs not necropsied. Myocardial disease was probably present in at least a portion of these, but was unattended by other clinically detectable signs of cardiac abnormality. Only once was an abnormal disturbance in rhythm or conduction seen in the absence of cardiac disease in the dogs necropsied.

It is well known that digitalis can both abolish and cause cardiac arrhythmias. In this series, abolition of atrial fibrillation can be cited as an example of the former effect. Atrioventricular dissociation was found only in dogs receiving digitalis glycosides. Some instances of ventricular premature systoles and ventricular parasystole can be attributed to digitalis.

Atrial arrhythmias were consistently associated with other signs of heart disease and were found in dogs with congestive heart failure more often than junctional or ventricular arrhythmias and conduction disturbances.

The following 3 reasons can be offered to

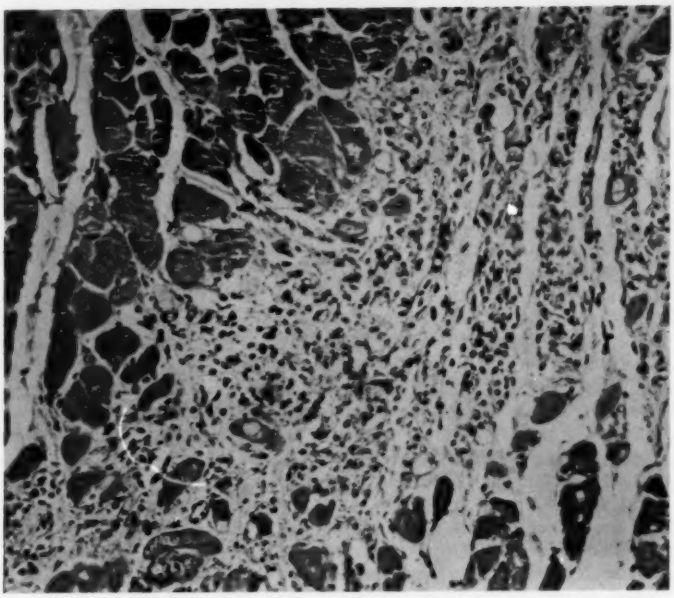

Fig. 7—Focal necrosis of the ventricular myocardium. Notice the loss of sarcoplasm and infiltration by lymphocytes and macrophages. H & E stain; x 90.

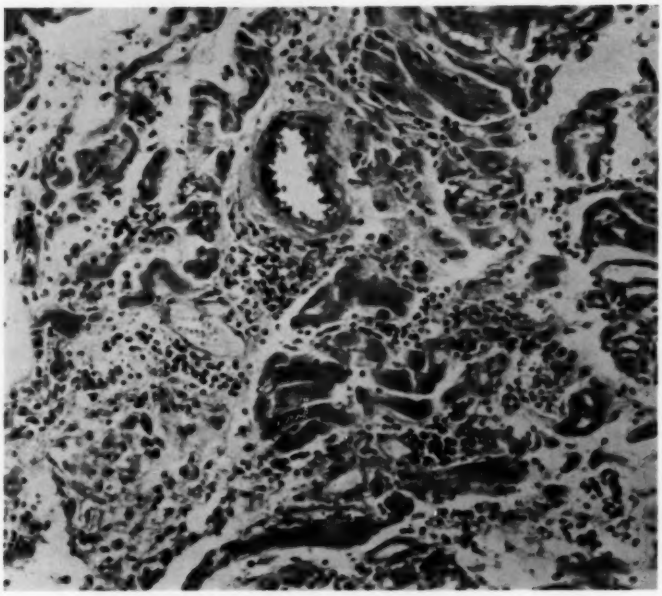

Fig. 8—Focal necrosis in the atrial myocardium of a dog with mitral valve fibrosis and insufficiency. Notice the disrupted fibers, edema, and accumulation of lymphocytes and macrophages. H & E stain; x 120.

explain the association between atrial arrhythmias and congestive heart failure: (1) Atrial fibrillation, atrial flutter, and atrial tachycardia result in inefficient cardiac activity. (2) In dogs with congestive heart failure, mitral insufficiency was commonly present and this lesion produced atrial strain, thus favoring atrial arrhythmias. (3) When chronic valvular disease was advanced enough to result in mitral insufficiency, myocardial disease involving both ventricular and atrial muscle was usually present.[9]

A few arrhythmias and conduction disturbances were well represented in the dogs studied clinically and postmortem, and can be discussed with regard to their clinical significance. In others, the clinical significance cannot be well defined.

Ventricular premature beats were by far the most common abnormality observed, and were associated with ventricular myo-

cardial disease in all dogs necropsied. Paroxysmal ventricular tachycardia, present also in 4 of the necropsied dogs which had isolated ventricular premature beats, likewise was associated with myocardial disease. Although ventricular premature beats are not considered definite signs of cardiac disease in man, this study supports the opinion that spontaneous, consistently occurring, ventricular premature systoles or paroxysmal ventricular tachycardia are reliable evidence of cardiac disease in the dog.

Atrial fibrillation and flutter are signs of severe cardiac disease in the dog, evidenced by the occurrence of congestive heart failure in all instances in this series. The underlying cause of these arrhythmias is apparently extensive atrial myocardial disease, associated in most cases with dilatation of the left atrium secondary to the mitral valve insufficiency of chronic valvular disease.

The limited clinical and postmortem studies reported here indicate that both incomplete atrioventricular block and incomplete atrioventricular block with dropped beats, although associated with heart disease in most instances, may occur in the absence of demonstrable cardiac disease.

Tracings closely resembling experimental right bundle branch block were associated with extensive right ventricular dilatation in 1 dog, and occurred in the absence of signs of cardiac disease in a 2nd dog. Both dogs with a left bundle branch block-like pattern had widespread myocardial disease. Recent experimental evidence [1] indicates that parietal focal block, in which lesions are located in the peripheral conducting tissue and myocardium of the ventricular walls, can simulate closely right or left bundle branch block. The occurrence of right bundle branch block in a dog with no other detectable cardiac abnormality suggests that this conduction disturbance, as in man, may be present in the absence of significant heart disease. The limited electrocardiographic studies made in the cases here described did not establish whether the conduction disturbances actually occurred in the main bundle branches or whether they represent more peripheral lesions.

Complete atrioventricular block can occur in adrenocortical insufficiency in the dog and may be reversed by the administration of adrenal corticosteroids and sodium chloride. This, as well as other electrocardiographic disturbances, may result from other than primary cardiac disease.

Electrocardiographic tracings of the type seen in Wolff-Parkinson-White syndrome have not been previously reported in the dog.

From postmortem studies, it is recognized that pathologic changes in the myocardium occur often without recognizable electrocardiographic disturbances. The frequency with which myocardial disease was present but did not result in arrhythmias or conduction disturbances has not been determined in our series.

SUMMARY

During a study of cardiovascular disease in a sample of 3,000 dogs presented to a large veterinary clinic, 124 spontaneous arrhythmias and conduction disturbances were observed in 95 dogs. Clinical means were used to study 61 dogs, while 34 were also studied by gross and histologic postmortem techniques. Abnormal arrhythmias and conduction disturbances were most often associated with signs of chronic valvular disease in the clinical group. In the majority of dogs necropsied, the electrocardiographic abnormalities were associated with chronic valvular disease, arteriosclerotic narrowing of the lumen of the small intramyocardial coronary arteries, and small foci of myocardial necrosis.

Arrhythmias and conduction disturbances were associated with inflammatory lesions of the myocardium, congenital heart disease, aortic body tumors, *Dirofilaria immitis* infection, and other conditions in a smaller number of animals.

A relationship between atrial arrhythmias and advanced chronic valvular disease was found. This association is explained by the extensive atrial myocardial changes which may occur in atrioventricular valve insufficiency.

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SUMMARIO IN INTERLINGUA

Spontanee Arrhythmias Cardiac e Disturbationes del Conduction Cardiac in le Can
Un Studio Clinic e Pathologic de 3.000 Canes

Durante un studio de morbo cardiovascular in un serie de 3.000 canes presentate a un grande clinica veterinari, 124 spontanee arrhythmias e disturbationes de conduction esseva observate in 95 canes. Medios clinic esseva usate in studiar 61 canes, durante que 34 esseva etiam studiate per technicas macro- e microscopic de post morte. Arrhythmias e disturbationes del conduction esseva observate le plus frequentemente in association con signos de chronic morbo valvular in le gruppo clinic. In le majoritate del canes in que studios necroptic poteva esser effectuate, le anormalitates electrocardiographic esseva associate con chronic morbo valvular, restringimento arteriosclerotic del passage del micre arterias coronari intramyocardial, e micre focos de necrose myocardial.

Arrhythmias e disturbationes del conduction esseva associate con lesiones inflammatori del myocardia, congenite morbo cardiac, tumores de corpore aortic, infection per *Dirofilaria immitis*, e altere conditiones in un plus micre numero de animales.

Esseva trovate un relation inter arrhythmias atrial e avantiate morbo valvular chronic. Iste association se explica per le extense alterationes atrio-myocardial que pote occurrer in le presentia de insufficientia del valvula atrioventricular.

A Quantitative Measurement of the Blood Loss Caused by Ancylostomiasis in Dogs

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THE AMOUNT of blood removed from the intestinal mucosa by the hookworm was studied quantitatively by Wells[6] in 1931. He collected a drop of blood from the anus of the parasite and counted the number of red blood cells contained in it. This red blood cell count was compared with that of a sample taken from peripheral blood. By this method, he was able to calculate the amount of blood represented by each drop. The number of drops occurring over a 22-minute period were counted. If the parasite continued to suck blood for 24 hours at the same rate as for the 22 minutes, then, each hookworm would withdraw 0.84 cc. of blood in 24 hours. Later, Nishi,[4] using similar methods, repeated Wells' work and arrived at a figure of 0.144 cc. per 24 hours for average blood consumption and 0.392 cc. per 24 hours for maximum blood consumption.

There were certain inherent errors in the research of Wells[6] and Nishi.[4] They measured the blood loss in only a few parasites (1 in the report by Wells) over a short period and extrapolated to 24 hours. In doing this, as Wells points out, one has to assume that the parasite sucks blood at a constant rate for 24 hours. In an attempt to study the amount of time each parasite spent sucking blood, he made observations on 200 worms. The length of the period of observations was not stated exactly, but apparently varied from a few minutes to an hour or 2. It was concluded that the parasites sucked almost continuously.

Recently, techniques for measuring gastrointestinal blood loss have been developed[2, 3, 5] using iron,[59] and chromium.[51] They are used to tag the red blood cells so that, when the blood passes through the parasite and into the feces, the radioactivity of the feces will be an index to the amount of blood lost.

Owen *et al.*[3] studied the reliability of using radiochromium-tagged red blood cells for measuring intestinal blood loss. It was their opinion that the test was reliable and the radioactivity of the feces closely paralleled the amount of blood lost. In one experiment, 92.1% of the blood administered orally was recovered from the feces. An additional 2.3% occurred in the blood and urine and the remainder was unaccounted for. Roche *et al.*[5] used radiochromium-tagged red blood cells to measure fecal blood loss in man and found it reliable.

MATERIALS AND METHODS

The general procedure in this experiment was to tag the red blood cells with radiochromium[51] and measure the amount of radioactivity that appeared in the feces of the dog. The total blood loss was computed by comparing the total radioactivity of the feces with the radioactivity of the circulating blood.

The animals used in this experiment were pups 6 to 10 weeks old from 4 separate litters. They were caged separately and fed a commercial dry dog food enriched with 10% dried milk and a complete mineral-vitamin supplement. The pups were obtained from bitches that were relatively free of parasites. All pups were free of parasites at the beginning of each experiment, except for one in which the postmortem examination revealed several tapeworms. Fecal flotation tests were made daily throughout the entire experimental period to evaluate the extent of parasitism. All dogs were dipped in a malathion solution and apparently were free of external parasites.

Procedure for Tagging and Counting Blood.—The red blood cells of all pups were tagged with radiochromium by a technique previously described.[1] In order to determine the level of radioactivity in the blood, samples were counted at 5-day intervals. This was done by withdrawing slightly over 1 cc. of blood from each pup. Exactly 1 cc. was pipetted into a test tube and its radioactivity determined.* This activity was plotted on semilogarithmic paper and the daily activity computed from these graphs.

Procedure for Determination of Fecal Radioactivity and Fecal Blood Loss.—All the feces for a 24-hour period were collected at the same time

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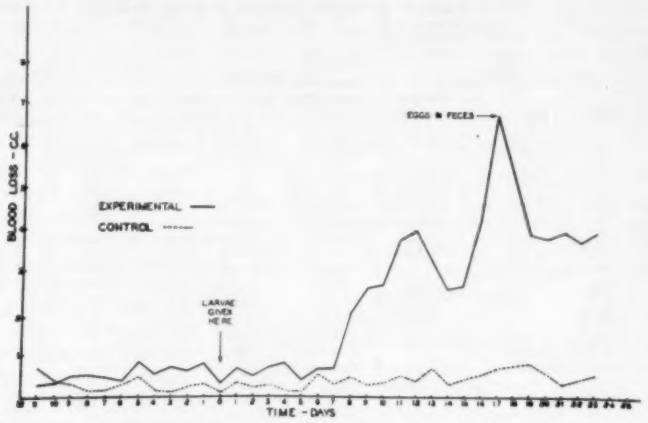

Fig. 1—Fecal blood loss from a typical experimental dog (No. 1) and a typical control dog (No. 2).

each day. Each 24-hour sample was thoroughly mixed with a minimum of water in a high-speed blender. The total volume of this mixture was recorded and 3 samples of 3 cc. each were prepared by using precalibrated test tubes. This volume was chosen to obtain maximum counts without losing activity due to the crystal geometry. The samples were counted in a deep well crystal connected to a single channel spectrometer and scaler.* Counts obtained from the individual samples were averaged, divided by the sample volume, and multiplied by the total fecal volume to obtain the total daily fecal radioactivity. The total amount of blood lost daily in the feces was computed by the following formula:

$$\frac{\text{Fecal activity}}{\text{Blood activity}} = \text{cc. of blood in feces}$$

Where:

Fecal activity = The total 24-hour radioactivity of the feces in counts per minute.

Blood activity = The radioactivity of 1 cc. of blood in counts per minute.

cc. of blood in feces = The cc.'s of blood lost in the feces in 24 hours.

When the red blood cells were tagged with radiochromium, a consistently small amount of the activity was found in the feces. This occurred even when the animals were apparently free of all parasitic and other diseases that might conceivably cause intestinal blood loss. During this investigation, it was not established whether this small amount of radioactivity was actual blood loss or whether it was simply a leakage of free chromium[51] into the intestine. When this radioactivity was calculated as blood loss, it amounted to slightly less than 0.5 cc. of blood per day (Table 1). The amount varied from animal to animal but was usually constant in the same animal. This normal daily loss of chromium[51] in the feces had to be taken into account when calculation of parasitic blood losses were made. This was done by subtracting the blood loss that occurred in a parasite-free animal from that which occurred after a parasite infection. The resultant value represented the blood loss caused only by the parasites.

These corrected figures were used to calculate both the mean and maximal daily blood losses. The mean daily blood loss was obtained by dividing the number of hookworms obtained from each animal at necropsy into the average daily blood loss for the entire experimental period. The maximal daily blood loss was obtained by dividing the hookworm load into the greatest blood loss that occurred on a single day during the experimental period.

The pups were allotted to experimental groups by litters. One or 2 members of each litter served as controls. This was done in order to keep to a minimum the individual variation among animals. The amount of blood lost in the feces was measured daily. The total blood loss was later compared to the total number of hookworms in order to compute the maximum and average amounts of blood lost per worm per day. After 7 to 15 days of normal measurements, each experimental animal was given between 100 to 200 hookworm larvae orally in a gelatin capsule. The daily measurements of blood loss were continued until the animals died or the experiment was terminated. A postmortem examination was performed on all animals, and the total number of parasites were counted and classified as to sex. At necropsy, 100 parasites obtained from the experimental animals were selected at random and classified as to species.

Studies were also conducted on the accuracy of the method for measuring blood in the intestine. This was done either by introducing, through a laparotomy, known quantities of radioactive blood into the intestine, or by giving it orally in a gela-

* Nuclear Chicago Spectrometer, Model 132 with a deep-well thallium-activated sodium iodide crystal, manufactured by Nuclear Chicago Corporation, Chicago, Ill.

TABLE 1—Blood Loss in Dogs as a Result of Hookworms

Dog	Blood loss (cc.) caused by hookworms per 24 hr.		Maximum blood loss (cc.) in 24 hr.	Mean blood loss (cc.) per parasite per 24 hr.	Maximum blood loss (cc.) per per parasite per 24 hr.	No. of hookworms	Blood loss (cc.) in parasite-free animals per 24 hr.	
	Mean	Standard deviation					Mean	Standard deviation
1	3.93	1.14	6.7	0.081	0.16	42	0.55	0.18
2	Control	.....	Control	Control	Control	0	0.28	0.13
3	3.57	1.27	5.24	0.057	0.09	58	0.26	0.14
4	5.0	1.88	9.6	0.0494	0.11	87	0.67	0.34
5	3.88	1.30	6.3	0.0814	0.145	43	0.37	0.24
6	8.87	3.70	16.5	0.0656	0.129	128	0.45	0.22
7	Control	.....	Control	Control	Control	0	0.31	0.14
8	4.32	1.53	6.75	0.04	0.071	95	0.54	0.26
BM	Control	.....	Control	Control	Control	0	0.77	0.45
BB	8.55	3.39	13.4	0.0648	0.106	126	0.37	0.24
BW	Control	.....	Control	Control	Control	0	0.44	0.12
FBW	9.15	2.49	10.9	0.085	0.109	100	0.6	0.29
BD	4.71	1.11	5.18	0.046	0.55	94	0.55	0.49
BWS	Control	.....	Control	Control	Control	0	0.48	0.3
BWE	Control	.....	Control	Control	Control	0	0.5	0.36
BF	7.1	3.90	11.0	0.071	0.11	100	0.5	0.3
Averages		.....	.....	0.07	0.12	.....	....	....

Average and maximum blood loss caused by hookworms in the dog are given in columns 1 and 2. Average and maximum blood loss caused by each parasite are given in columns 3 and 4. Values were obtained by dividing the total number of parasites (column 5) into the average and maximum values of columns 1 and 2. The last column gives the loss of chromium^{51} computed as blood loss in parasite-free animals.

tin capsule. The amount of radioactivity which appeared in the feces was then counted. The amounts of blood given varied from 1 to 10 cc. These amounts were chosen because they were nearly the amounts of blood lost through parasitism by the dogs in these experiments.

RESULTS

Most of the pups began losing blood as a result of the hookworms on the 8th day after receiving the larvae orally. One pup began losing blood on day 7 and 2 on day 9. The amount of blood lost daily continued to increase until it reached a peak 12 to 13 days after the larvae were given. Blood losses then decreased on the 15th day and increased to a 2nd peak by day 17 or 18 (Fig. 1). After this, small oscillations in the daily blood loss continued to occur throughout the experimental period.

The worms began to discharge eggs on day 14 and 15 after the larvae were given orally. These eggs were detectable by fecal flotation methods on the 15th and 16th days. It required 24 hours for eggs placed in the small intestine to appear in the feces.

The total number of parasites in each animal was recorded (Table 1). It was found that 90% of the worms were *Ancylostoma caninum* and 10% were *Ancylostoma braziliense*. Of these, 51.3% were females and 48.7% were males.

The hosts' average blood loss per day for each hookworm was 0.07 cc. The mean value for maximum daily blood loss per hookworm was 0.12 cc.

In those experiments designed to test the accuracy of the entire procedure, it was possible to recover in the feces more than 90% of the activity contained in the blood given orally (Table 2).

TABLE 2—The Efficiency of Detection of Radiochromium-Tagged Blood in the Feces; Recovery of Blood After Administration of Varying Amounts by Different Routes

Dog	Amount of blood given (cc.)	Route	Recovered in feces*
			(%)
1	5	Oral	92.5
2	5	Oral	100.0
3	2	Oral	99.7
4	5	Direct intestinal injection.	94.0
5	5	Direct intestinal injection.	98.0
6	10	Oral	96.3
		Mean	96.7

* All figures corrected for the amount of chromium normally lost through the intestine.

DISCUSSION

The figures obtained for average daily blood losses per parasite were much lower than those obtained by Wells [6] and slightly lower than those reported by Nishi.[4] This discrepancy can easily be understood when consideration is given to the different manner in which the measurements were obtained. Wells and Nishi measured the blood loss over a short period when the parasites

were actually sucking blood. When the entire population of hookworms in each of our dogs was examined at necropsy, it was found that not all the parasites were engorged with blood or even attached to the intestinal mucosa. This was in contrast to the observation of Wells that most worms were constantly sucking blood. It is quite likely that a hookworm will attach and suck large quantities of blood for a short period and then either detach or cease to suck blood. This would result in a lower over-all 24-hour average than measurements made during periods of maximal blood removal.

Also, Wells and Nishi measured the blood lost through the female parasite whereas the present studies involved almost equal numbers of males and females. There is some possibility that females may suck more blood than males, especially during periods of egg laying.[5]

When blood sucking began, on about the 8th day, the amount of blood lost rose rapidly to a peak at approximately the 12th or 13th day. Blood loss then fell to a lower level on day 15 and reached a 2nd peak on day 18 (Fig. 1). There is some possibility that the 4th stage larvae suck blood, causing the 1st peak. During molting of the larvae to the adult stage, a low in blood sucking occurs about the 12th or 13th day. From then on, blood loss is due to the adult.

Roche *et al.*[5] studied the blood loss caused by hookworms in man, using the radiochromium-tagged red blood cell technique. Their results indicate that *Necator americanus* in man sucks approximately 0.0311 cc. of blood per day per worm. This value is less than that recorded for Ancylostoma species in the dog. This might be explained on the assumption that there is some species difference in the blood-sucking abilities of the 2 parasites.

SUMMARY AND CONCLUSIONS

1) The measurement of blood loss in the intestines by tagging the circulating red blood cells with chromium^{51} is a reliable technique. By its use, it was possible to measure the amount of blood loss as a result of Ancylostoma infection in dogs.

2) Each parasite removed a mean of 0.07 cc. of blood per day and a maximum of 0.12 cc. of blood per day. Within the limits of this experiment, the number of hookworms infecting an animal did not affect the amount of blood sucked by each worm.

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SUMMARIO IN INTERLINGUA

Un Mesuration Quantitative del Perdita de Sanguine Causate per Ancylostomiasis in Canes

1. Le technica de mesurar le perdita de sanguine in le intestinos per marcar le erythrocytos circulante con chromo^{51} es un technica de alte fidelitate. Per medio de illo il esseva possibile mesurar le perdita de sanguine resultante in canes ab infection per ancylostoma.

2. Le parasito individual prendeva un quantitate medie de 0,07 cm^3 de sanguine per die e un quantitate maximal de 0,12 cm^3 de sanguine per die. Intra le limites del presente experimento, le numero del vermes inficiente un animal non afficeva le quantitate de sanguine sugite per le verme individual.

Comparative Study of Feline Cytopathogenic Viruses and Feline Panleukopenia Virus

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A NUMBER of viruses that are cytopathogenic in tissue culture have been isolated from cats in the past few years. Although many of these viral agents were obtained from the upper respiratory tract and conjunctivas, 3 were isolated from the spleen. Studies of the serologic relationships of the feline cytopathogenic viruses have been reported previously.[2-4] In the present report, the disease-producing potential and antigenic relationships of the 3 viruses isolated from the spleens of cats are compared with each other and with panleukopenia virus. Evidence of an apparent synergistic effect in dual infections with panleukopenia virus and one of the tissue culture viruses is described.

MATERIALS AND METHODS

Viruses.—The viruses studied were FPL,* originally isolated by Bolin[2]; KCD,** originally isolated by Fastier[5]; F-20†; and feline panleukopenia.‡

The F-20 virus was designated as FJ in a previous report.[3] The FPL, KCD, and F-20 viruses were propagated in tissue culture. Since standard feline panleukopenia virus has not been sucessfully propagated in tissue culture, the source of the virus used in these studies was a 10% suspension of spleen from panleukopenia-infected cats.

Tissue Cultures.—The cortical tissue of kidneys obtained from 8- to 12-week-old kittens was trypsinized by a method previously described by Youngner.[6] A 0.5% cell suspension was made in a growth medium consisting of 0.5% lactalbumin hydrolysate and 10.0% horse serum in Earle's basic salt solution, penicillin (200 units/ml.), and streptomycin (200 μg./ml.). This was dispensed in 1.0-ml. amounts into tubes which were kept stationary until good growth was observed (usually 5 to 7 days). The fluid was then replaced, using 2 ml. of a maintenance medium similar to the growth medium, except that the serum concentration was 5.0%. The tubes were then placed in roller drums.

Neutralization Test.—Antiserums were prepared against FPL, F-20, and KCD viruses by twice-weekly intradermal inoculation in rabbits, for a period of 3 weeks, with 1.0 ml. of live, undiluted tissue culture virus. Panleukopenia antiserum was obtained from cats that had recovered from the disease.

The neutralization test was performed by preparing tenfold dilutions of test serum, and mixing an equal volume of virus with each dilution in a final concentration calculated to contain 100 $TCID_{50}$ per 0.2 ml. The serum-virus mixtures, as well as virus in control tubes, were incubated for 2 hours at room temperature, after which 0.2 ml. of each dilution was inoculated into each of 4 feline kidney tissue culture (FKTC) tubes. The tubes were incubated at 35 C. and read at 2 and 7 days. The 50% dilution end point per 0.1 ml. of serum was calculated by the formula of Reed and Muench.[8]

Experimental Animals.—Litters of 4 or more 6- to 10-week-old kittens were obtained from farms in the surrounding area, placed in isolation, and observed for 3 to 4 weeks. The susceptibility of each kitten to the tissue culture viruses was established by determining that specific neutralizing antibody was not present in the serum. Individual litters were presumed to be susceptible to panleukopenia virus when the immunity of 1 littermate kitten was challenged and signs of panleukopenia developed.

Cross-Protection and Pathogenicity Studies.—Cats were inoculated to determine the disease-producing potential of each of the 3 tissue culture viruses, and to compare these results with Fastier's findings for KCD virus. The cats were inoculated intramuscularly with 1.0 ml. of virus, except that in one experiment 0.25 ml. of FPL virus was inoculated intranasally.

To further compare FPL and F-20 viruses with panleukopenia virus, cross-protection studies were made in litters of 4 or more cats which were allotted in equal numbers to separate isolation rooms. Half of the cats in each litter were inoculated with either FPL or F-20 virus. The others were

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The authors thank Dr. James W. Newberne for performing the pathologic examinations in the panleukopenia and dual infection studies, and Dr. Karl Mayer for editorial assistance in the preparation of this report.

* Supplied through the courtesy of Major R. A. Crandell, Armed Forces Institute of Pathology, Washington, D.C., as FPL-FM t.c. No. 3, July 3, 1958.

** Supplied through the courtesy of Dr. L. B. Fastier, Tasman Vaccine Laboratories, Upper Hut, New Zealand, as KCD t.c. No. 14, July 23, 1958.

† Isolated by J. B. Emery, Virus Research Laboratory, Pitman-Moore Co., Division of Allied Laboratories, Inc., May 19, 1954.

‡ Standard challenge strains, Biological Laboratories, Pitman-Moore Co., Division of Allied Laboratories, Inc.

inoculated with panleukopenia virus. The immunity of surviving cats was cross-challenged.

RESULTS

Characterization of Agents.—In studies to characterize FPL, F-20, and KCD, all 3 agents gave essentially similar results.

All were cytopathogenic in feline kidney tissue culture, with destruction of the monolayer cell sheet in 24 to 48 hours; virus titers ranged from $10^{5.5}$ to $10^{7.5}$ per 0.2 ml. No loss in virus titer occurred after storage for 1 year at −70 C. Tissue culture fluids retained their infectivity for FKTC after passage through a Selas 03 filter.

TABLE 1—Cross-Neutralization Studies with 3 Feline Viruses Isolated from Spleen

Viruses	$TCID_{50}$	Antiserums			
		FPL	KCD	F-20	Panleukopenia
FPL	100	1:10,000	Neg.*	Neg.	Neg.
KCD	100	1:10	1:5,000	1:320	Neg.
F-20	64	Neg.	1:50	1:20,000	Neg.

* Negative titers represent no neutralization with 1:10 serum dilution.

Infective tissue culture fluids of each virus, inoculated into the yolk sacs of 7- to 8-day-old embryonating chicken eggs, produced no deaths; no elementary bodies were found in yolk sac smears stained by Machiavello's method. No overt signs of disease were produced when suckling and adult Swiss Webster mice were inoculated intraperitoneally or intracerebrally with each virus, and no growth was observed in thioglycollate or PPLO mediums inoculated with the tissue culture fluids. No hemagglutination occurred with feline, guinea pig, or rabbit erythrocytes at 4 C. and 37 C.

Serologic Studies.—The results of cross-neutralization experiments to determine the serologic relationships of the 3 tissue culture viruses and panleukopenia are summarized (Table 1).

The FPL virus was not neutralized by KCD, F-20, or panleukopenia antiserums. However, FPL antiserum in low dilution neutralized KCD virus.

The KCD and F-20 viruses were neutralized by their respective homotypic and heterotypic antiserums, although not to the same degree. When the formula of Archetti and Horsfall [1] was applied to the results, a significant antigenic difference between these 2 viruses was shown.

Panleukopenia convalescent serum did not neutralize any of the 3 tissue culture viruses.

Cross Protection.—Cats that had recovered from FPL or F-20 virus infection responded typically to experimental panleukopenia infection after challenge with that virus. Conversely, cats that had recovered from panleukopenia infection developed signs of FPL or F-20 virus disease when their immunity was challenged (Tables 2, 3).

Experimental Infection—FPL Virus.—Of 20 cats initially inoculated with FPL virus in 5 separate experiments, all proved to be susceptible and developed fever, depression, and anorexia within 24 to 48 hours. Temperatures ranged from 104 to 105 F. and persisted for an average of 5 days. Depression and anorexia were present during the period of hyperexia. A mild leukopenia of 1 to 2 days' duration also occurred. The body temperature and leukocyte responses are depicted (Fig. 1). With the exception of 1 kitten that died in litter 2, recovery was rapid and no chronic signs were observed in cats held for several months after inoculation. Cats exposed by cage contact developed similar signs after a slightly longer incubation period (3 to 4

TABLE 2—Cross-Protection Studies with FPL and Panleukopenia Viruses

Litter No.	No. of cats	Panleukopenia after FPL				FPL after panleukopenia			
		Inoculated with FPL virus		Immunity challenged with panleukopenia virus		Inoculated with panleukopenia virus		Immunity challenged with FPL virus	
		Sickness	Death	Sickness	Death	Sickness	Death	Sickness	Death
1	8	4/4*	0/4	4/4	1/4	4/4	1/4	3/3	0/3
2	8	8/8	1/8	2/2	0/2	.....	.....	.....	.....
3	4	2/2	0/2	2/2	0/2	2/2	0/2	2/2	0/2
4	4	4/4	0/4	.....	.....	.....	.....	.....	.....
5	4	2/2	0/2	2/2	0/2	2/2	0/2	2/2	0/2
Total	28	20/20	1/20	10/10	1/10	8/8	1/8	7/7	0/7
Per cent		100	5	100	10	100	12.5	100	0

* Numerator = number of cats reacting; denominator = number of cats tested.

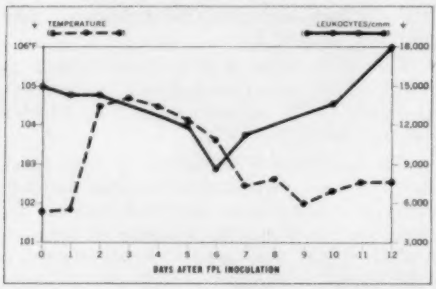

Fig. 1—Average body temperature and leukocyte response of 4 cats inoculated with FPL virus.

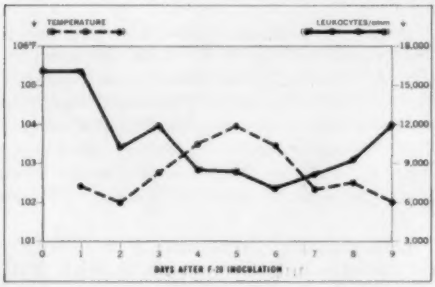

Fig. 2—Average body temperature and leukocyte response of 3 cats inoculated with F-20 virus.

days). Convalescent serum titers ranged from 1:3,200 to 1:100,000.

Infections with FPL were shown to be present in the Indianapolis area when serum of cats from 2 different sources was tested. Approximately 18% (2 of 11) of farm-raised cats over 1 year old had FPL antibody titers greater than 1:100; whereas, 65% (11 of 17) of cats of approximately the same age, obtained from a commercial vendor, had antibody titers of greater than 1:100.

F-20 Virus.—The F-20 virus produced a disease syndrome similar to, but usually milder than, that caused by FPL infection. Hyperexia, physical depression, and anorexia were the main signs. A transient leukopenia (Fig. 2) also occurred. No mortality resulted in 10 cats given an initial inoculation of F-20 virus. Convalescent serum titers ranged as high as 1:32,000. Apparently, F-20 is also common in the Indianapolis area, since a high percentage of the cats obtained locally had pre-existing antibody titers.

KCD Virus.—Failure to produce any overt signs of disease with KCD virus, reported by Fastier,[5] was confirmed in a limited number of cats in this laboratory. Of 2 kittens inoculated intramuscularly with KCD virus, neither developed clinical signs. Pre-inoculation KCD serum antibody titers from these cats were less than 1:5; whereas, serum titers 3 weeks after inoculation were 1:800 and 1:3,200.

Fastier did not observe any gross variation in leukocyte count. He also showed that approximately 50% of apparently normal cats over 18 weeks of age had KCD antibody titers of 1:100 or greater.

Panleukopenia Virus.—These strains of panleukopenia virus consistently produced typical leukopenia, depression, anorexia, bone marrow aplasia, and intestinal lesions in susceptible cats. The body temperature responses and occurrence of leukopenia are shown (Fig. 3). The mortality rate was 10%; 3 of 30 cats from 9 different litters died when given challenge doses of this strain of virus.

Dual-Infection Studies.—In 3 separate experiments, intramuscular injections of 1.0 ml. of FPL virus and 1.0 ml. of panleukopenia virus were given simultaneously to each of 11 susceptible cats. The body temperature response occurred in 24 hours and remained elevated throughout the course of the disease. Anorexia and physi-

TABLE 3—Cross-Protection Studies with F-20 and Panleukopenia Viruses

Litter No.	No. of cats	Panleukopenia after F-20				F-20 after panleukopenia			
		Inoculated with F-20 virus		Immunity challenged with panleukopenia virus		Inoculated with panleukopenia virus		Immunity challenged with F-20 virus	
		Sickness	Death	Sickness	Death	Sickness	Death	Sickness	Death
6	4	4/4*	0/4	4/4	1/4	.....	.....	.....	.....
7	3	3/3	0/3	3/3	0/3	.....	.....	.....	.....
8	3	3/3	0/3	3/3	0/3	.....	.....	.....	.....
9	2	.....	.....	.....	.....	2/2	0/2	2/2	0/2
Total	12	10/10	0/10	10/10	1/10	2/2	0/2	2/2	0/2
Per cent		100	0	100	10	100	0	100	0

* Numerator = number of cats reacting; denominator = number of cats tested.

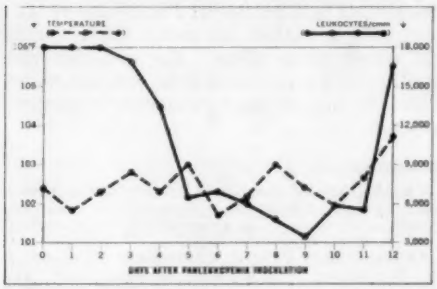

Fig. 3—Average body temperature and leukocyte response of 4 cats inoculated with panleukopenia virus.

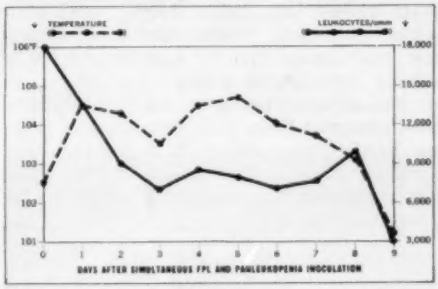

Fig. 4—Average body temperature and leukocyte response of 6 cats inoculated simultaneously with FPL and panleukopenia viruses.

cal depression became apparent on about the 3rd day of infection, and lasted throughout its course. The leukocyte count dropped rapidly, from an average of 18,000 to 8,000, and remained at about this level until the 8th day, when a more severe leukopenia developed (Fig. 4). Of the 11 cats given challenge inoculations in this manner, 9 died between the 9th and 10th day after inoculation.

Signs of the dual infection were more pronounced and the mortality rate (82%) was considerably higher than that produced by either virus alone.

Discussion

Results of the studies made to characterize FPL, F-20, and KCD demonstrate that these cytopathogenic agents isolated from spleen are viruses and are antigenically different, although some degree of cross-relationship appears to exist, especially between F-20 and KCD viruses. Evidence indicating a slight relationship between the agents isolated from spleen and those isolated from the upper respiratory tract and conjunctivas has been reported previously, and it would be premature to place these viruses in separate groups according to the tissues from which they were isolated.

Pathogenicity studies indicate a wide variation between the 3 tissue culture viruses isolated from the spleen. The FPL virus produced acute signs of short duration; 1 of 27 cats died after challenge with FPL virus. The F-20 virus produced a similar syndrome which, however, consistently appeared to be less severe; KCD virus did not cause any overt signs. The length of time that these viruses are harbored in the cat was not determined, but the high incidence of neutralizing antibody in adult cats indicates that the viruses are widespread.

The FPL, KCD, and F-20 viruses, also compared serologically and by cross-protection studies with 2 strains of panleukopenia virus, were shown to be unrelated to the panleukopenia strains used. The latter consistently produce typical leukopenia, depression, and anorexia, as well as bone marrow aplasia and typical intestinal lesions, in susceptible cats. The fact that the FPL virus of Bolin was not related to panleukopenia in these tests may be due to several reasons. However, in view of the fact that virulent panleukopenia is easily transmitted, the most likely possibility is that in the original work, both FPL and panleukopenia viruses might have been present simultaneously in the experimental cats.

That the panleukopenia virus used in these experiments was free of any contamination with FPL, F-20, or KCD viruses was demonstrated by repeated failure to isolate the latter agents following inoculation of these viruses in tissue culture, and by the absence of specific neutralizing antibodies for FPL, F-20, and KCD in feline convalescent serum after inoculation with the panleukopenia virus. The mortality (10%) with these strains of panleukopenia virus is low in comparison with the mortality of 66 to 83% described in other reports.[6,7] An attempt was made to explain this difference by studying the effects of dual infections. A more severe syndrome, accompanied by high mortality (82%) and produced by the simultaneous administration of FPL and panleukopenia viruses, suggests that the 2 viruses act synergistically. The severe experimental disease approximates the per-

acute disease often seen during panleukopenia epizootics. Preliminary studies suggest that other viruses also may have a similar synergistic effect. Variations in clinical panleukopenia mortality rates have been observed from year to year, and from one locality to another. It is not yet clear whether this variation is due to this synergism with other viruses, or to differences in strain virulence.

SUMMARY

Three viruses (FPL, F-20, and KCD), cytopathogenic in feline kidney tissue culture and obtained by isolation from spleens of cats, were studied. Experimentally, FPL produced fever of about 5 days' duration; transient mild leukopenia, depression, and anorexia. Recovery was rapid. The F-20 virus produced a similar but much milder illness, and KCD produced no overt signs. Partial antigenic relationship was shown between F-20 and KCD viruses; there was no antigenic relationship between any of the 3 cytopathogenic viruses and panleukopenia. Serologic evidence indicated that FPL and F-20 are common infections in cats in the localities tested. Simultaneous inoculation of FPL and panleukopenia into susceptible cats produced a more severe disease with a higher mortality (82%) than did either virus alone. The experimental dual infection appeared to be comparable to that seen in some field epizootics of panleukopenia.

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SUMMARIO IN INTERLINGUA

Studio Comparative de Viruses Cythopathogenic Felin e Virus de Panleucopenia Felin

Esseva studiate 3 viruses (designate como FPL, F-20, e KCD). Illos esseva cytopathogenic in culturas de tissu renal felin e esseva obtenite per isolation ab splenes de catto. Experimentalmente, FPL produceva febre de un duration de circa 5 dies, transiente leucopenia de leve grados, depression, e anorexia. Le restablimento esseva rapide. F-20 produceva un simile morbo, sed isto esseva multo plus leve. KCD produceva nulle signos patente. Un leve grado de relation antigenic esseva demonstrate inter FPL e KCD. Esseva trovate nulle relation antigenic inter ulle del tres viruses cytopathogenic e panleucopenia. Evidentia serologic indicava que FPL e F-20 es infectiones commun in cattos in le locos testate. Le inoculation simultanee de FPL e panleucopenia in susceptibile cattos produceva un morbo plus grave con un plus alte mortalitate (82%) que le un o le altere del suo sol. Il pareva que le dual infection experimental esseva comparabile a illo vidite in certe epizootias de panleucopenia in le campo.

Pendulous Crop in the Chicken—A Pathologic Study

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PENDULOUS CROP, commonly found in the turkey but seldom in the chicken, was produced experimentally in the turkey by Wheeler *et al.*[4] The anatomical and pathologic changes in the turkey with a spontaneously occurring pendulous crop were described by Rigdon *et al.*[2] In the latter study, it was shown that the primary lesion was focal necrosis of the striated muscle. The pathologic study of a chicken with a spontaneously occurring pendulous crop, reported here, revealed the same type of muscular degeneration as was present in the turkey.

A White Leghorn chick, hatched on February 1, 1960, in the laboratory of Dr. Morris Pollard at the Medical Branch in Galveston, Texas, was from a group of eggs obtained from the Poultry Department of the Agricultural and Mechanical College of Texas. This chicken had "curly toes" at the time of hatching. No other abnormality was seen. It was kept with a group of normal chicks of the same age for the first 2 weeks, after which time it was individually caged.* The crop was found to be enlarged on the 67th day after hatching. The enlargement increased until the 74th day, at which time the bird (Fig. 1) was brought to the laboratory for pathologic study. It weighed 890 Gm., and the crop was definitely enlarged. There was regurgitation of fluid when the head was lowered.

The bird was killed, and the skin removed to expose the enlarged crop (Fig. 2). The mucosa lining the crop was normal both grossly and microscopically. Grossly, a few petechiae were present in the muscles of the thigh and of the pectoral area (Fig. 2). No changes were found in the viscera either grossly or microscopically. The nervous system was not studied. Multiple sections were removed from the striated muscles in different portions of the body. These were fixed in a 4% solution of formaldehyde. Paraffin sections were prepared and stained routinely with hematoxylin and eosin.

Fig. 1—Photograph showing pendulous crop in a chicken 74 days old.

Focal areas of Zenker-like necrosis were found microscopically in many of the sections of muscles (Fig. 3). The necroses involved only small segments of individual muscle fibers. Usually, the involved portions of muscle were swollen and stained deeply with eosin. Sometimes, a sharp line of demarcation distinguished the necrotic

Fig. 2—Carcass of chicken shown in Figure 1 after feathers were removed. Arrow indicates location of a group of petechiae in muscle.

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From the Department of Pathology, University of Texas, Medical Branch, Galveston.

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* The chicken was fed Purina Chick Growena, a product made by Ralston Purina Food Co., St. Louis, Mo.

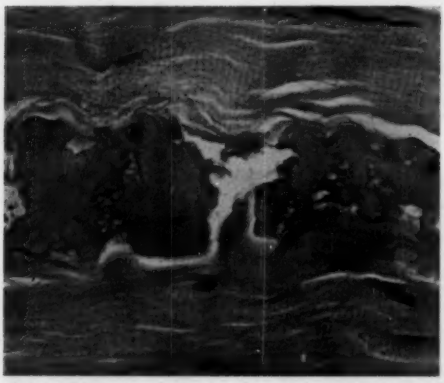

Fig. 3—Focal area of Zenker-like necrosis in striated muscle of the leg. H & E stain; x 475.

area from the adjacent normal muscle fiber. Focal collections of red blood cells were present in some of the sections of muscle (Fig. 4). Several muscle fibers in a focal area were degenerated. The remains of the muscle wall in this area suggested sarcolemma-like tubes (Fig. 5). In one area of muscle, there was a large collection of lymphocytes.

The pathologic changes in the striated muscles of this chicken were identical to those present in the turkey with pendulous crop. In the latter, the necrosis occurred in the striated muscle supporting the crop, as well as in striated muscle elsewhere in the body. It has been suggested that pendulous crop in the turkey is a manifestation of weakness, resulting from the degeneration of the muscle. A similar mechanism may account for pendulous crop in the chicken.

Necrosis of the striated muscle has been described by Dam *et al.*[1] in chickens fed a vitamin E-deficient ration. Morphologic changes in the muscle of the chicken with pendulous crop were similar to those observed by Dam in vitamin E-deficient birds. We have no information on the vitamin E content of the commercial ration consumed by this chicken. White Pekin ducks in our laboratory have extensive Zenker-like necrosis in the striated muscle. Some of these birds have shown contractures.[3]

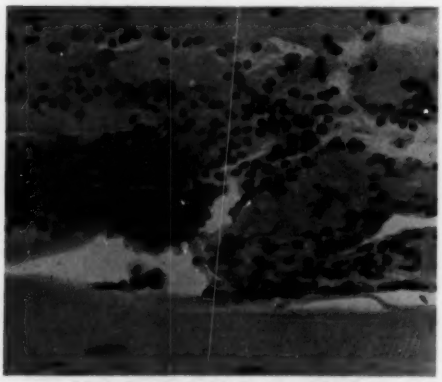

Fig. 4—Focal area of red blood cells in area of degenerated muscle from leg. H & E stain; x 410.

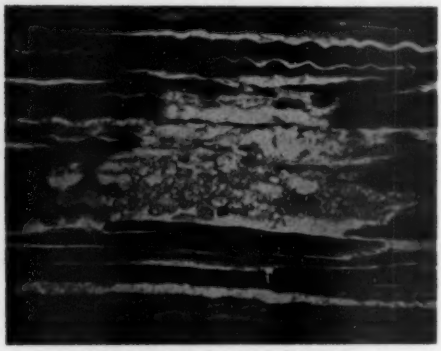

Fig. 5—Focal area of degeneration of several fibers in pectoral muscle. H & E stain; x 123.

SUMMARY

Pendulous crop occurred spontaneously in a chicken hatched and raised in the laboratory. It was fed a commercial ration. Acute Zenker-like degeneration was present in focal areas of striated muscle of the legs and pectoral region. These changes in the muscle were similar to those previously observed in turkeys with pendulous crops. The cause is not known; however, the problem is being studied.

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SUMMARIO IN INTERLINGUA

Ingluvies Pendule in Gallinas—Un Studio Pathologic

Un pendule ingluvie occurreva spontaneemente in un gallina covate e elevate in le laboratorio. Acute degeneration, del typo de degeneration de Zenker, esseva presente in areas focal de musculo striate in le gambas e le region pectoral. Iste alterationes muscular esseva simile al alterationes previemente reportate in gallos de India con ingluvies pendule. Le causa non es cognoscite. Tamen, le problema es sub studio.

A Study of *Salmonella typhimurium* Infection in Market-Age Turkeys

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EXTENSIVE SALMONELLA contamination of equipment and carcasses during processing of poultry has been conclusively demonstrated by Browne [2] and Galton *et al.*[4] Some evidence of the probability that infected carcasses will be processed on any given day has been reported by Sadler *et al.*[11] Shedding of Salmonella in feces of artificially infected young birds was demonstrated by Gauger and Greaves,[6] who reported that poults shed *Salmonella typhimurium* in feces up to 96 days after oral infection. Shaffer *et al.* [12] reported recovery of *S. typhimurium* from feces for at least 18 days after oral infection of baby chicks, and Adler *et al.*[1] recovered it from feces and tissues up to 44 days after oral infection of 6.5-week-old poults. Gauger and Greaves [5] also reported fecal shedding of this organism up to 65 days by naturally infected adult turkeys; artificially infected adult turkeys did not shed it after the 15th day, although it was present in the liver and cecal mucosa of 1 of the birds on the 23rd day. Gibbons and Moore [7] quantitated the fecal shedding of *Salmonella bareilly* by adult chickens naturally infected with *S. pullorum* and artificially infected *per os* with the test organism. They reported a maximum of 19,500 organisms per gram of feces in 1 of 6 birds, with a maximal length of shedding at 38 days. There appears to be little information on the duration of infection or shedding, or both, in market-age birds or on the numbers of organisms in their feces. The numbers shed may be quite large if at all comparable to the 10^4 to 10^6 per gram found by Thomson [13] in the feces of symptomless, human carriers.

The present experiment was conducted to determine the pathogenicity of *S. typhimurium* for market-age turkeys, its distribution and number in tissues and intestinal contents, the numbers shed in the feces, and the serologic response of the birds to systemic or intestinal infections, or both.

MATERIALS AND METHODS

Inoculum.—The organism used was recovered from the intestinal tract of a turkey (field case) and was typed as *S. typhimurium* at the California State Department of Public Health Laboratories. A broth stock culture was inoculated intraperitoneally into day-old poults. The organism was recovered on MacConkey agar from the liver of a dead poult. Single colonies were seeded into beef heart infusion broth and tested for purity by routine methods. One milliliter of an 18-hour, 2nd broth culture passage, containing 1.2×10^9 organisms, was instilled into the crop of each bird.

Turkey Inoculation.—Twenty-two 11-month-old turkeys of both sexes and mixed breeding (Broad Breasted Bronze and Beltsville Whites), initially negative for Salmonella by serology and culture, were allotted to 2 groups of 3 and 19. The primary objective of the experiments conducted with group 1 was to determine the rate of fecal shedding of the organism following infection. The birds were placed in separate cages in an isolation cubicle. One of the birds was given an iron oxide capsule [8] so that time of passage of the inoculum through the intestinal tract could be approximated. Feed and water were given to the birds without restriction. Droppings falling through the bottom screen onto clean brown wrapping paper were collected at intervals. The cages were cleaned and the bottom screen changed at weekly intervals. The first sampling consisted of a composite of cecal and intestinal feces. For samples taken at subsequent intervals, an attempt was made to collect intestinal and cecal feces in separate containers. These were labeled and placed in the refrigerator until quantitative counts could be made (2 to 8 hours after sampling). Blood samples were obtained at 14 and 21 days after inoculation, and the samples were tested using the method of McNeil and Hinshaw [9] for *S. typhimurium* agglutinins. The birds were killed at 21 days, and the presence and number of organisms were determined in various organs, feathers, dust, and feed.

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The experiment with birds of the larger group (group 2) was designed to supplement information gained from the 1st group. These birds were inoculated in a similar manner and placed on litter in an open cubicle. Eggs were collected for 14 days after inoculation. Blood samples were then obtained, and cloacal swabs were taken for culture. Blood samples were obtained and the birds were killed in 3 groups at 35, 41, and 44 days after inoculation, and various tissues were taken for quantitative Salmonella study.

Quantitative Methods.—The fecal samples were weighed in 0.5- or 1.0-Gm. quantities, and serial tenfold dilutions were made in quarter-strength Ringer's diluent. Two methods of counting were employed.

In the first method, utilizing the most probable numbers (MPN) technique, proper dilutions of the samples were inoculated into selenite-cystine broth [10] in triplicate. The broth cultures were plated on brilliant-green agar after 18 to 24 hours of incubation at 37 C. The colonies that developed on the agar were identified in a manner which will be described later. The MPN, based on 3 tubes per dilution, was determined from the tables of Buchanan and Fulmer.[3]

The second method (drop-plate technique) consisted of inoculations of 0.01 ml. of a dilution of sample onto a brilliant-green agar plate. Five replicates were used per dilution; usually 3 dilutions were plated per sample. The agar plates were incubated 24 hours at 37 C., and the number of Salmonella colonies were counted in each drop from any one dilution. With some experience it was not difficult to recognize Salmonella colonies on brilliant-green agar. When dilutions of the sample were inoculated onto this medium, discrete colonies (glistening, semitransparent, and lactose-negative) could be readily recognized as presumptively belonging to the Salmonella group. Further definitive identification was made by picking colonies for the rapid slide-agglutination test, using diluted, specific, *S. typhimurium* antiserum. Further confirmation was made by inoculation of triple sugar iron agar and urea slants. Since a culture of *S. typhimurium* was used initially in these experiments, the method was felt to be sufficiently accurate to identify the recovered organisms. A few of the isolates recovered from various sources were sent to the California State Public Health Laboratory for serologic typing.

Although the drop-plate method is essentially a direct-counting method, it was also possible to calculate MPN from data obtained (based on the MPN tables and using 5 samples per dilution).

Besides the quantitative studies, inoculations were made directly into selenite broth for plating to brilliant-green agar as a routine procedure.

Results and Discussion

Group 1.—Iron oxide was found in fecal droppings 3.5 to 4.5 hours after crop instillation. To determine the proportion of inoculum shed during this initial period, all droppings for the first 5 hours were collected, pooled, and mixed thoroughly with a spatula. Total weight of feces shed during this period was determined for each bird. In addition, 1-Gm. portions were weighed for quantitative studies. During this initial period, bird 1 shed 1.19×10^8 organisms (10% of the original inoculum), and bird 3 shed 1.71×10^6 organisms (0.14%). Technical difficulties prevented accurate determination of Salmonella in the feces of bird 2; from the data obtained, however, the estimate was that fewer than 10^5 organisms were voided (0.008% of the original inoculum).

The numbers of Salmonella voided at various intervals after crop instillation are shown (Fig. 1–3).

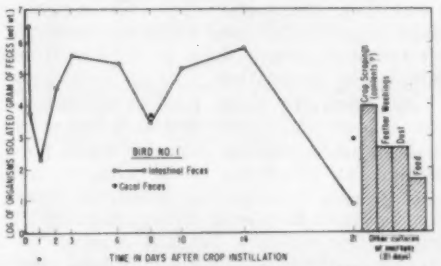

Fig. 1—Numbers of Salmonella typhimurium in feces excreted on indicated days after crop instillation, and numbers in feces, tissues, and environment upon termination at 21 days.

The counts are recorded on the basis of 1 Gm., wet weight, of feces. Calculations based on dry weights gave essentially the same relative difference in numbers between

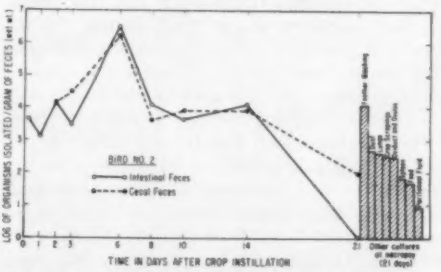
LOG OF ORGANISMS ISOLATED/GRAM OF FECES (wet wt.)
BIRD NO. 2
Intestinal Feces
Cecal Feces
TIME IN DAYS AFTER CROP INSTILLATION
Other cultures of carcasses (21 days)

Fig. 2—Numbers of Salmonella typhimurium in feces excreted on indicated days after crop instillation, and numbers in feces, tissues, and environment upon termination at 21 days.

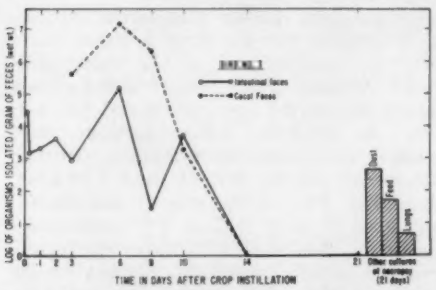

Fig. 3—Numbers of Salmonella typhimurium in feces excreted on indicated days after crop instillation, and numbers in feces, tissues, and environment upon termination at 21 days.

cecal and intestinal feces. The moisture content of feces from the 3 birds during the first 72 hours was about 35%; afterward, it was in the range 40 to 65%. The increase could be attributed most likely to change in environment rather than to disease from Salmonella inoculation.

The relatively large number of organisms voided during the initial 5 hours by birds 1 and 3 probably indicates that a portion of the inoculum, along with normal food, passed through the intestinal tract. The numbers decreased during the first 24 hours, after which a gradual increase occurred, reaching a peak about 6 days after inoculation. In birds 1 and 2, the numbers remained at a relatively high level until 14 days. By 21 days, they had decreased to a low level. In bird 3, however, numbers dropped sharply after a peak at 6 days; by 14 days no organisms were detected in either cecal or intestinal feces. Birds 1 and 2 had more organisms in cecal feces than in intestinal feces at 21 days. These results typify recovery rates at later intervals from the 18 birds in group 2. As is evident in the reported data that follow, some birds in group 2 yielded positive cultures from both cecal and intestinal feces, others were positive only from the cecal feces, and still others yielded negative cultures from both intestinal and cecal feces (Fig. 4).

The 3 birds were killed 21 days after inoculation, and the following organs and materials were taken for culture and enumeration: liver, kidneys, muscle, lungs, oviduct and ovaries, spleen, crop scrapings, cecal feces, intestinal feces, feather washings, dust, and feed. The positive cultures are listed (Fig. 1–3).

There were no clinical signs and the birds were in good condition. At necropsy, birds 1 and 3 had no gross lesions, but bird 2 had ascites and pneumonia. As shown (Fig. 2), cultures were positive, in decreasing order of relative numbers, from feather washings, cecal feces, lungs, crop contents, oviduct and ovules, spleen, and peritoneal fluid. Counts were based on 1-Gm. wet-weight samples except for feather washings; the feathers were washed with Ringer's diluent, and the amount of solids present in 1 ml. of the washing was calculated.

Bird 1 yielded the organism, in decreasing order of relative numbers, from the crop, cecal feces, feather washings, and intestinal feces. Bird 3 yielded the organism only from the lungs, which appeared normal at necropsy. Dust and feed from the cubicle also yielded the organism, which may reflect the presence of the organism in the crops and lungs of these birds.

At 14 days, only bird 1 had an agglutination titer of 1:25. At 21 days, all birds had titers of 1:25 (though the reading was questionable, complete, and partial for birds 1, 2, and 3, respectively).

Group 2.—As mentioned earlier, this portion of the experiment was terminated at 44 days. To facilitate the work, however, the birds were killed in small groups at 35, 41, and 44 days. Material and several tissues were taken for culture, though quantitative determinations were made only on samples found positive by routine culture. Two days were usually sufficient to determine whether a sample would be positive. Holding interval did not influence the viability of the organisms to any extent, as will be shown in another report.[14] Quantitative determinations made on cecal and intestinal

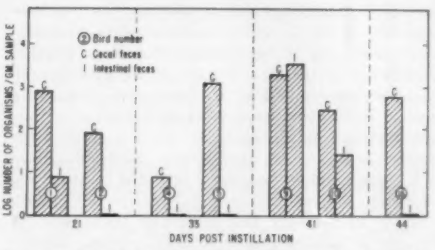

Fig. 4—Numbers of Salmonella typhimurium in intestinal and cecal feces of 7 turkeys on indicated days after crop instillation.

TABLE 1—Distribution of Salmonella typhimurium in Tissues of 18 Turkeys Fed 10^5 Organisms 35 to 44 Days Prior to Necropsy

Bird No.	Material cultured*											
	Liver	Lungs	Spleen	Ovaries, oviduct	Feather washings	Crop	Cecal feces	Intestinal feces	Cloacal swabs 14 days	Cloacal swabs Necropsy	Serotiters** 14 days	Serotiters** Necropsy
2–1	−	−	−	−	−	−	−	−	−	−	≧20†	40
2–2	+	+	+	−	−	−	−	−	+	−	<20	40
2–3	−	−	−	−	−	−	+	−	+	−	<20	80
2–4	−	−	−	−	+	−	+	+	+	−	≧20	20
2–5	−	−	−	+	+	−	+	−	−	+	≧20	40
2–6	−	−	−	−	+	+	+	+	+	+	<20	<20
2–7	−	−	−	−	+	−	+	+	+	−	<20	40
2–8	−	−	−	−	−	−	−	+	+	−	≧20	40
2–9	−	−	−	−	−	−	−	−	+	−	<20	80
2–10	−	−	−	−	−	−	−	−	+	+	<20	<20
2–11	−	−	−	−	−	−	−	−	+	+	<20	<20
2–12	−	−	−	−	−	−	−	−	+	+	≧20	80
2–13	−	−	−	−	−	−	+	−	+	−	≧20	<20
2–14	−	−	−	−	+	−	−	−	+	−	<20	40
2–15	−	−	−	−	−	−	+	−	+	−	<20	<20
2–16	−	−	−	−	−	−	−	−	+	−	≧20	20
2–17	−	−	−	−	−	−	−	−	+	−	≧20	<20
2–18	−	−	−	−	+	−	−	−	−	−	ND	40
Total positive	1	1	1	1	6	1	7	4	15	5	8	12

* Approximately ¼ Gm. of minced tissue was inoculated into at least 2 sets of enrichment medium; *i.e.*, duplicate sampling for each tissue examined. Kidney, muscle, and bile, also cultured from each bird, were negative. ** Agglutination titers given as reciprocal of + dilution. † Screened at 1:20 only.

feces from these birds are summarized (Fig. 4). Determinations for samples from birds in group 1 are also listed for comparison. In general, cecal feces yielded more organisms than did corresponding intestinal feces. It was not uncommon to have positive cecal feces with negative intestinal feces from the same bird.

At necropsy, all birds appeared to be in good condition. Gross lesions were not evident in any of them. Among 18 turkeys, 1 yielded Salmonella from liver, lungs, and spleen, and another yielded the organism from the ovaries. The spleen and liver, respectively, yielded 500 and 5,000 organisms per gram of tissue; the lungs and ovaries yielded fewer than 10 organisms per gram of tissue.

TABLE 2—Isolation of Salmonella typhimurium from Feces of 18 Turkeys as Determined by Source Sample

Combination	No. of samples positive in combination: No.	Combination	No.	Combination	No.
IF+		IF+		CF+	
CF+	3	CS+	1	CS+	2
IF+		IF+		CF+	
CF−	1	CS−	3	CS−	5
IF−		IF−		CF−	
CF+	4	CS+	4	CS+	3

PERCENTAGE OF ISOLATIONS

CF	7/18(38.9%)
IF	4/18(22.2%)
CS	5/18(27.8%)
CF or CS, or both	10/18(55.5%)
CF or IF, or both	8/18(44.5%)
CS or IF, or both	8/18(44.5%)

IF = intestinal feces; CF = cecal feces; CS = cloacal swabs.

TABLE 3—Comparison of Isolation of Salmonella typhimurium with Serologic Response in 18 Turkeys

Method of isolation	Time (days) after feeding culture: 14 (CS)	35–44* (CS)	35–44* (CS, CF, IF, and/or V)
+ isolation + serology	6(33.3%)**	2(11.1%)	7(38.8%)
+ isolation − serology	9(50.0%)	3(16.7%)	5(27.8%)
− isolation + serology	3(16.6%)	10(55.5%)	5(27.8%)
− isolation − serology	0(0%)	3(16.6%)	1(5.6%)
Totals	18	18	18
Total + isolation	15	5	12
Total + serology	9	12	12

CS = cloacal swabs; CF = cecal feces; IF = intestinal feces; V = visceral organs (*see* text for method of culture); + isolation and + serology = positive isolation and serology (titer ≧ 1:20).

* Necropsy. ** Number and percentage of birds.

The results of Salmonella isolation and the serologic response elicited at 14, 35, and 44 days after inoculation are shown (Table 1). The cloacal swab method yielded 83.5% (15/18) isolations at 14 days, and 27.8% (5/18) at the later time. When data from all 4 methods of culture were tabulated without duplication, however, 67% (12/18) of the birds harbored the organism at one

site or another at the later time. These results indicate that the numbers of *S. typhimurium* decreased with time, making recovery more difficult. The results (Table 2) demonstrate that isolations were more frequent when cultures were taken from several sources in the intestinal tract. The growth medium used also influenced recovery rate.[14]

Positive correlation of isolation results and serologic findings (Table 3) showed no substantial increase between the 1st and 2nd dates (33.3% as opposed to 38.8%).

Samples of litter were taken at random at 14 days. Quantitative determination on 3 aliquots indicated that some 10,000 organisms were present per gram of litter. At the end of the experiment, 44 days after oral inoculation, samples of feed, litter, and water were taken for quantitative study. The number of organisms per gram was fewer than 20 in feed, fewer than 10 in water, and fewer than 40 in litter. A direct culture of feed, water, and litter yielded Salmonella only from the last. The low counts in the environment may explain the low recovery from the crop contents (Table 2).

Surface Contamination of Eggs in Relation to Shell Penetration.—Eighty-two eggs were collected during a 14-day period after inoculation of the hens. They were laid on the litter, and no attempts were made to keep the surfaces free from feces or dust. Feces, feathers, and dust were scraped from 15 eggs for quantitative determination of surface contamination. The scrapings were divided into 3 parts, and counts made by the MPN technique. Final calculation gave an estimate of 74 organisms contaminating each egg. As mentioned earlier, the litter at this time contained 10,000 organisms per gram. The other 67 eggs collected were incubated at 39 C. for 5 days before culture. All embryo namples were negative.

SUMMARY

Twenty-one adult turkeys were inoculated in the crop with 1.2 x 10^9 *Salmonella typhimurium* originally isolated from the intestinal tract of a turkey. The rate of fecal shedding was determined in 3 birds over a 21-day period. There appears to be a difference in numbers present in the cecal and intestinal feces, the former generally giving higher counts. Various organs and material were cultured at 21 days for quantitative study. Experiments with the remaining 18 birds showed that 83.4% of the birds were positive by the cloacal swab method at 14 days. At necropsy, 35 to 44 days postinoculation, 27.8% of the birds were positive by the same method, but the total of positives was increased to 67% when all methods of culture (swab, direct fecal, and visceral organs) were considered. The positive correlation between serologic findings and isolation results was 33.6% at 14 days and 38.8% at necropsy. Although the organism was recovered from the surface of the eggs, subsequent culture of incubated eggs yielded negative results. On culture of the various internal organs from the 21 birds, 1 bird yielded the organism from liver (5,300 per gram), spleen (550 per gram), and lungs (less than 9 per gram). Positive material was obtained from the ovaries of a 2nd bird (fewer than 8 per gram); from the lungs of a 3rd turkey (fewer than 10 per gram); and from the lungs (10^3 per gram), oviducts (10^3 per gram), spleen (10^2 per gram), and peritoneal fluid (10 per milliliter) in a 4th bird. Kidney and muscle tissues were negative. Positive fecal cultures at necropsy yielded from 10 to 10^3 organisms per gram.

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SUMMARIO IN INTERLINGUA

Un Studio de Infection per *Salmonella typhimurium* in Gallos de India de Etate Mercabile

Vinti-un adulte gallos de India esseva inoculate in le ingluvie con 1,2 x 10^5 de *Salmonella typhimurium* originalmente isolate ab le vias intestinal de un gallo de India. Le proratas del elimination fecal esseva determinate in tres aves in le curso de un periodo de 21 dies. Il pare exister un differentia inter le numeros del organismos presente in le feces cecal e le feces intestinal. In le prime, le numerationes esseva generalmente plus alte. Varie organos e materiales esseva culturate post 21 dies pro studios quantitative. Experimentos con le remanente 18 aves monstrava que 83,4% de illos esseva positive secundo le methodo a tampon cloacal post 14 dies. In le necropsias, inter 35 e 44 dies post le inoculation, 27,8% del aves esseva positive secundo ille mesme methodo, sed le total del positivitate montava a 67% quando omne le methodos de culturation esseva prendite in consideration (tampon, fecal directe, e organos visceral). Le correlation positive inter le constatationes serologic e le resultatos del isolation esseva 33,6% post 14 dies e 38,8% al tempore del necropsia. Ben que le organismo esseva retrovate al superficie del ovos, le subsequente culturation de ovos incubate habeva resultatos negative. In le culturation de varie organos interne del 21 aves, 1 del aves monstrava le organismo in le hepate (5.300 per g), in le splen (550 per g), e in le pulmones (minus que 9 per g). Material a positivitate esseva obtenite ab le ovarios de un secunde ave (minus que 8 per g), ab le pulmones de un tertie (minus que 10 per g), e ab le pulmones (10^3 per g), le oviductos (10^3 per g), le splen (10^2 per g), e le liquido peritonee (10 per ml) in un quarte. Ren e tissu muscular esseva negative. Le positive culturas fecal al necropsia rendeva 10 a 10^5 organismos per g.

A Phage-Typing System for Staphylococci from Turkeys with Synovitis

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STAPHYLOCOCCAL SYNOVITIS of fowl has been recognized for many years in many species. In adult Broad Breasted Bronze turkeys it is a costly infection, killing up to 20% of some flocks of birds 9 weeks of age or older.

This synovitis is apparently a primary infection. From killed affected birds, large numbers of *Staphylococcus aureus* can be isolated from the hock, wing, and sternal synoviae which undergo pathologic change, varying from fulminating purulence to barely perceptible changes. At necropsy, *Staphylococcus aureus* can also be isolated routinely from liver, spleen, kidneys, and blood. The liver is the most common source, and the lesions vary from near normal appearance to mahogany color and necrotic areas. The kidneys are frequently swollen.

Intravenously reinjecting these staphylococci into healthy turkeys consistently produces the complete syndrome. The source of the causative Staphylococcus remains unknown as do the portal of entry, the mechanism of invasion, and other basic phenomena.

Discovery of the source may be simplified by the typing of *S. aureus*. A system for typing the isolates from eggs; from healthy, dying, and dead turkeys; from other animals in the turkey environment, including man; and from feed, water, and fomites may indicate the source or sources of staphylococci.

Bacteriophage typing of staphylococci has won wide acceptance as the best, but far from perfect, method of detecting differences between strains.[1, 7, 8] Serologic typing of staphylococci in our laboratories has not proved useful.

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MATERIALS AND METHODS

To develop a bacteriophage system for typing the staphylococci isolated from turkeys and their environments, we first considered the 48 bacteriophages sent us by Fisk.* Of these, 27 were isolated by Fisk[3] from staphylococci of human origin, and 21 were from the Wilson and Atkinson[4] collection. At this time, we also received 4 staphylophages and their propagating strains from Blair.** Finding these 52 staphylophages excessively cumbersome, we arbitrarily selected 4 Fisk phages (10/56, 541/531, 541/536, and 544/542), and 6 Wilson and Atkinson phages (3B, 3C, 6, 7, 44A, and 44B).

The first several staphylococci from turkeys tested with these bacteriophages responded uniformly to phage 44A and were resistant to the other staphylophages.

The typing method used for these studies was essentially that of Fisk.[3] The phage was used as produced as a test concentration. Minimum lytic doses of phage were not determined. We were probably using more than 1,000 times the present routine test concentration. In these first studies, only complete lysis was accepted as a criterion of the susceptibility of the staphylococci. The 10 selected phages were tested against 72 coagulase-positive, mannitol-fermenting, gelatin-liquefying, and hemolytic (on defibrinated sheep blood in veal infusion agar) staphylococci from 13 moribund or dead turkeys. Twelve of the turkeys yielded staphylococci which were susceptible to 44A and resistant to the other phages.

Attention was turned next to developing a phage system that might detect differences among these 44A-sensitive staphylococci. Using in a few cases the method of Smith,[6] but chiefly that of Rountree,[5] 27 44A-sensitive staphylococcal isolates were studied for the presence of other staphylophages. Of these staphylococci, 23 were isolated from dead or dying turkeys, 3 from mice caught in turkey pens, and 1 from the soil of a turkey yard.

The methods of phage purification, cultivation, titration, and, in some cases, original isolation were largely those of Smith.[6] The individuality of these phages was established by testing them against 27 strains of staphylococci from turkey sources and by cross-resistance studies. Several

* Roy T. Fisk, The Hyland Laboratories, Los Angeles, Calif.

** John E. Blair, Hospital for Joint Diseases, New York, N.Y.

were similar to each other. The differences, however, were consistent and reproducible, though slight. Nine purified staphylophages were selected for use, plus bacteriophage 44A (of the international system) which was grown in our laboratory on "turkey" staphylococcus strain J16 and was therefore designated 44AT (T for turkey) at the suggestion of Blair.[3] The new phages and their propagating strains were designated "J" phages and strains for Jensen and James, the two authors more closely associated with this phase of our program.

These 10 phages were tested against 79 cultures of *S. aureus* freshly isolated from infected turkeys. These represented 40 turkeys in 15 flocks, located in 4 geographical areas separated by distance and, in some instances, by high mountains. More than 1 culture was isolated from some of the birds. The distribution of staphylophage patterns of isolates from the 40 turkeys is shown (Table 1).

1 strong reaction were assigned to the same pattern. Where an apparent intermediate lay midway between 2 patterns, the assignment was made to the larger group which was nearest geographically. Thus, errors in judgment, if any, were not in the direction of the conclusions of this paper.

The data suggest that this phage-typing system can be used to type a substantial proportion of staphylococci isolated from turkeys with synovitis in Utah. Only 8 of 40 turkeys yielded nontypable strains.

Nine staphylophage patterns occurred within this small group of cultures (Table 1). Most patterns differing by only 1 or 2 reactions were assigned to the nearest key pattern. Thus, more than 9 typable strains may be present.

TABLE 1—Distribution of Staphylococcus Strains Obtained from Turkeys Within Geographic Areas

Staphylophage pattern	Geographic areas*				Total No. of birds
	I	II	III	IV	
J1/J1A/J1B/J5/J18/J21/J21A/44AT	3	8	..	1	12
J1/J1B/J5/J18/J21/44AT	..	2	..	..	2
J1/J1B/J5/J12A/J18/J27/44AT	..	6	1	..	7
J1B/J5/J18/44AT	..	1	..	..	1
J1A/J1B/J21A/44AT	..	1	1	..	2
J1A/J5/44AT	..	1	..	..	1
J12A/J18/J27/44AT	1	..	3	..	4
44AT	2	..	..	..	2
J1	1	..	..	..	1
Nonsensitive (nontypable)	..	..	..	8	8
Total	7	19	5	9	40

* I = Box Elder County in the northern part of Utah; II = Cache County, east and adjacent to I, separated by a mountain range from other areas; III = Davis County, 20 miles south of I; IV = Sanpete County, 150 miles south of I and separated by mountains from other areas.

Results and Discussion

By choosing the phages that caused confluent lysis in the staphylococci from 1 or more turkeys, patterns were set up. For example, 12 turkeys (1st pattern, Table 1) yielded strains which were lysed by bacteriophages 44AT/J1/J1A/J1B/J5/J18/J21/J21A, and 7 "turkey" staphylococcus strains (3rd pattern, in Table 1) differed from this by resistance to J1A/J21/J21A and by susceptibility to J12A and J27. Thus, these 2 patterns differed from each other in 5 different "strong" lytic reactions.

According to Williams and Rippon,[7] where 2 strong reaction differences exist, a difference in strain is presumed to exist. They define a strong reaction as one causing complete lysis, confluent lysis, or more than 50 plaques. Strains differing by only

It was strongly indicated that there is a regional distribution of strains. Even though the dominant strain occurred in 3 of the 4 areas studied, on the basis of this sample it appears that, within limits, each geographic area had its own strains.

Jensen[4] has also tabulated these results by flock. The evidence is suggestive that flocks not widely separated may have synovitis caused by different Staphylococcus strains.

Summary

Nine staphylophages were obtained from staphylococcal cultures isolated from turkeys, mice, and a soil sample. These new phages, plus bacteriophage 44A, detected marked and consistent differences among staphylococcal strains isolated from turkeys with synovitis.

Epizootics of staphylococcal synovitis in

turkeys within a geographic area, such as a mountain valley, were found to be caused by several strains of *S. aureus* different from strains occurring in distant areas.

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SUMMARIO IN INTERLINGUA

Un Systema de Phagotypia pro Staphylococcos ab Gallos de India con Synovitis

Novem staphylophagos esseva obtenite ab culturas de staphylococcos isolate ab gallos de India, muses, e un specimen de terra. Iste nove phagos, insimul con bacteriophago 44A, detegeva marcate e uniforme differentias inter racias de staphylococcos isolate ab gallos de India con synovitis.

Esseva trovate que epizootias de synovitis staphylococcic in gallos de India intra un area geographic—un valle in le montanias, per exemplo—es causate per plure racias de *Staphylococcus aureus* que differe ab le racias occurrente in areas distante.

Focal Necrosis in the Mucosa of the Gallbladder in Pigs with Hog Cholera

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CONSIDERABLE DATA have been compiled on the pathologic changes produced in pigs with hog cholera virus. However, a comprehensive microscopic analysis of some of the lesions produced in the course of the disease has yet to be made.

It is the purpose of this report to describe and discuss lesions characterized by focal necrosis of the mucosa of the gallbladder, and the experimental conditions under which they were observed during a 3-year period.

REVIEW OF LITERATURE

Ivanov *et al.*[2] reported from Bulgaria on the macroscopic lesions of the gallbladder and cystic ducts of 163 pigs experimentally infected with swine fever virus and 13 spontaneously infected pigs. They also reported on the microscopic lesions found in 67 of the gallbladders and 45 of the cystic ducts. The main lesions present 4 to 6 days after infection were destruction of lymphatic tissue, formation of pseudomembranes, hemorrhage, and hyperemia. Hemorrhage and hyperemia were visible macroscopically in 18 and 64% of the animals and microscopically in 39 and 84%, respectively. The pseudomembranes appeared to be analogous to the early stages of ulcers in the large intestine.

In previous necropsy reports in experimental investigations, Dunne *et al.*[3] observed mild to severe petechial hemorrhages of the gallbladder. Quin[5] mentioned that fine petechiae were found on the epiglottis and on the mucosa of the gallbladder.

One of the 5 most significant diagnostic signs of African swine fever, as reported by Mauer *et al.*,[4] was congestion and edema of the gallbladder. Grossly, engorgement of the superficial blood vessels was the most striking change. The wall of the gallbladder was edematous. At times, the edema extended to the liver capsule, making it appear thickened and gelatinous along the line of attachment. Petechiae and ecchymoses frequently were scattered over the serous and mucosal surfaces. The gallbladder was usually distended with bile. Microscopically, there was vascular engorgement, submucosal edema, karyorrhexis of lymphocytes in the submucosa, and small hemorrhages.

MATERIALS AND METHODS

The pigs used in this study weighed approximately 25 to 40 lb. Most of them were raised from gilts or sows susceptible to hog cholera. Some of the pigs were raised at the university, while others were purchased from local farmers. The major breed used was Yorkshire, although other breeds and crossbreds were used.

The pigs included in this study were those that died or were killed when in a moribund condition resulting from experimental infection with hog cholera virus.

The gallbladder observations were made on 106 pigs exposed to several types of hog cholera virus from tissue cultures or virulent virus in pathogenesis studies. Virus from 6 lots, representing 2 sources, was used to challenge the immunity of pigs surviving experimental exposures.

When areas of necrosis were observed at necropsy, they were recorded as mild, moderate, or severe, depending upon their number and size. Bile characteristics were recorded as thick, solid, watery, dark, granular, and no bile; gallbladder size was recorded as enlarged, shrunken, or normal.

Several representative photographs were made

Fig. 1—Representative infarctions of gallbladders from pigs experimentally infected with hog cholera virus.

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Fig. 2—Severe infarctions in the gallbladder of a pig infected with hog cholera virus.

before and after fixation in 10% formalin. The sections were stained with hematoxylin and eosin. The lungs (whenever pathologic changes were observed) and spleens were cultured for pathogenic organisms.

RESULTS

Of 265 experimentally infected pigs, dead or killed in a moribund condition, 106 had macroscopic lesions. Forty-eight had severe lesions (represented by A912, 1038, 1039, and 1083 in Fig. 1), 19 had moderate lesions (represented by A1009 and 943), and 39 had mild lesions (represented by A1020, 1003, and 1058).

A severe lesion prior to excision from the liver is shown (Fig. 2). A closer view of a lesion from another severely affected gallbladder after fixation in formalin is shown (Fig. 3). The wall of this particular gallbladder was considerably thickened.

Of the pigs with gallbladder lesions, 54.0% had illness lasting 11 to 20 days; 14.5%, more than 40 days; 13.5%, 21 to 30 days; 12.5%, 10 days or less; and the remainder (5.5%) were sick 31 to 40 days.

In 85% of 79 cases recorded, the bile was dark, thick, and granular; in 9%, the bile was normal; and in 6%, the bile was watery.

Of 69 gallbladders, 34 (49%) were small; 19 (27%) were normal; and 17 (24%) were enlarged.

Adult ascarids were observed in 16 of 106 pigs with gallbladder lesions. In 43 of the 106, there were scars on the liver due to ascarids.

Correlating the areas of focal necrosis in the gallbladder with lesions of the large intestine, 30 of 106 pigs had severe button ulceration of the colon or cecum, and 6 had moderate, 23 had mild, and 47 had no enteric button ulceration. Five of the latter 47 pigs had necrotic enteritis; 9 had hemorrhagic enteritis.

Bacteriologic examination of the lungs (whenever pathologic changes were present) and of the spleens showed 51% to be bacteriologically negative. Bacteria were cultured from 26% of the spleens and 32% of the lungs. The spleens contained 14 bacterial species; the lungs, 16.

Microscopically, the areas of necrosis in the gallbladder appeared to be analogous with areas of acute infarction reported as being present in the large intestine. The blood vessels at the base of necrotic areas had hydropic swelling of the endothelial cells with a marked narrowing of the lumens of the vessels, resulting in a stasis of the blood in the area. Also, there was a sparsity of recognizable blood vessels in the base of the infarcted area.

An area of coagulation necrosis in the

Fig. 3—Severe infarctions in the gallbladder of a pig (A685) infected with hog cholera virus. The thickened wall is also shown.

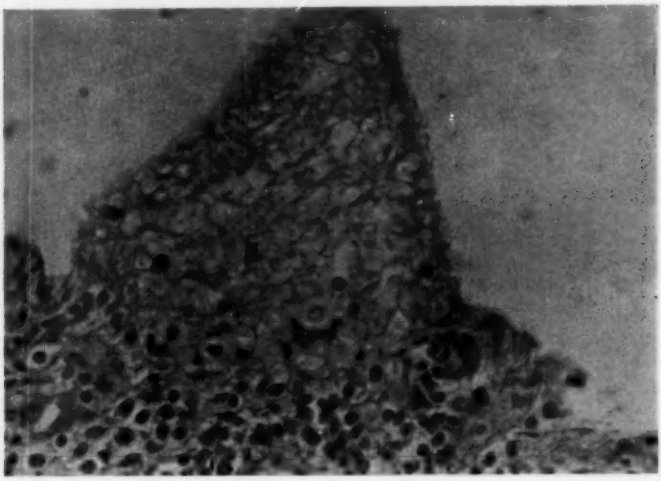

Fig. 4—Coagulation necrosis of a tip of one of numerous irregular folds of the mucosa-submucosa of the gallbladder. The simple columnar epithelium has been eroded. H & E stain; x 258.

mucosa in which the columnar epithelium was eroded is shown (Fig. 4). Also shown is an ulcer extending to the tunica muscularis with considerable leukocytic invasion at the border of the necrotic tissue and living tissue (Fig. 5). The later stages in which the necrotic tissue was raised above the level of the mucosa, having the appearance of a pedicle, is shown (Fig. 6, 7). Mucus and cellular debris frequently adhered to the pedicle, adding to its raised appearance. This coincides with the gross lesion that is frequently observed. In many of the sections, there was considerable vascular engorgement and small hemorrhages, especially in the lamina propria. In some sections in which the wall was greatly thickened, the thickness was due to hyperplasia of all layers; in others, it was due primarily to an increase of the perimuscular connective tissue. For these reasons, the lesions will be described hereafter as areas of focal necrosis resulting from infarction.

Discussion

The relation of infarction to formation of "button" ulcers in pigs with hog cholera has been reported by Dunne *et al.*[1]

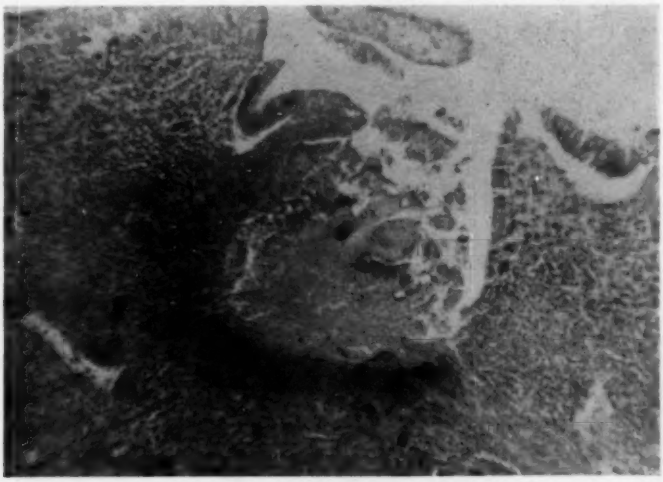

Fig. 5—Area of coagulation necrosis of the mucosa-submucosa at an early stage of ulcer formation. Notice the heavy, well-devoloped line of leukocytic infiltration in the submucosa. H & E stain; x 96.

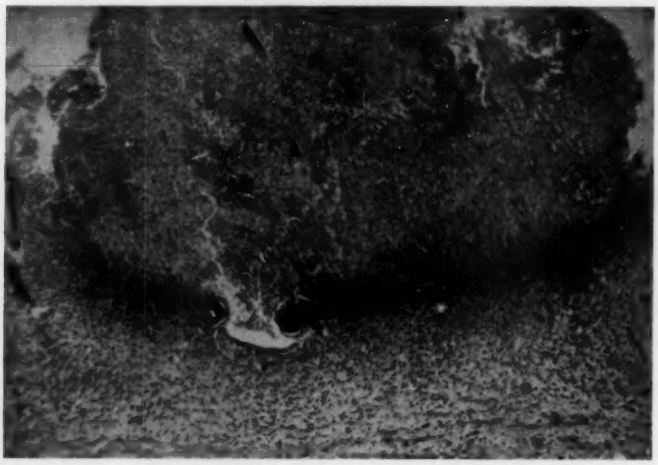

Fig. 6—Infarction showing associated villi, and coagulation necrosis. Notice the marked leukocytic line. H & E stain; x 100.

They also reported that button ulceration was one of the oldest recognized lesions. However, the histologic changes and pathogenesis of the gallbladder lesion had not been described. Examinations of pigs with field cases of hog cholera over a period of years have revealed a number of the lesions which we now describe as areas of infarction in the gallbladder. In most instances, these were mistaken for hemorrhages. The lesions described herein were not observed in test control pigs or in other pigs submitted for necropsy but not infected with hog cholera virus. The negative bacteriologic findings in 51% of the spleens and lungs suggest that septicemia is not associated with the gallbladder ulcer. The only common factor appears to be hog cholera virus.

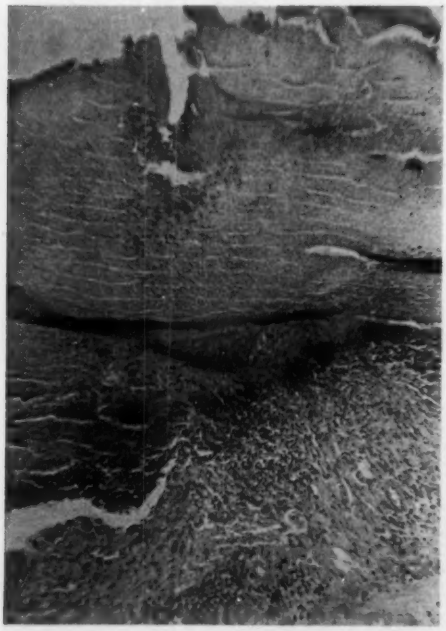

Fig. 7—A later stage of the infarction. Notice the pedicle, the leukocytic line, and the elevated necrotic plug. H & E stain; x 91.

The correlation of the button ulcers in the large intestine with areas of focal necrosis in the gallbladder shows that in 59 pigs both were present, while gallbladder lesions occurred in 5 pigs with necrotic enteritis and in 9 pigs with hemorrhagic enteritis. The interesting fact is that in 33 pigs, areas of necrosis were present in the gallbladder but not in the large intestine. Even though this occurred more frequently in animals with the acute form of the disease, it also occurred in some pigs with subacute and chronic hog cholera. The button ulcer of the large intestine has been considered by many to be pathognomonic for hog cholera. The results of these studies suggest that areas of infarction in the mucosa of the gallbladder are also pathognomonic, thus providing another diagnostic aid.

Enlarged gallbladders occurred in only 25% of the pigs, and the majority of these occurred in pigs with the acute form of the

disease. However, 2 occurred in pigs with the prolonged chronic form and others occurred in pigs with the subacute form. In African swine fever, the gallbladder is usually distended, but in hog cholera it is more commonly shrunken.

SUMMARY

1) Areas of infarction were observed in 106 (40%) of the gallbladders from 265 pigs experimentally infected with hog cholera virus.

2) None of the secondary bacterial invaders isolated appeared to have influenced the occurrence of areas of infaction. The only common factor known was hog cholera virus.

3) The areas of infarction in the gallbladder appeared to be comparable with those observed and reported in the large intestine and were believed to be pathognomonic for hog cholera.

4) In addition to the areas of focal necrosis in the gallbladders of pigs experimentally infected with hog cholera virus, infarcted areas also were observed in pigs with hog cholera that were submitted to the diagnostic laboratory.

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SUMMARIO IN INTERLINGUA

Necrosis Focal in le Mucosa del Vesica Biliari de Porcos con Cholera Porcin

1. Areas de infarcimento esseva observate in 106 (=40 pro cento) del vesicas biliari ab 265 porcos experimentalmente inficite con virus de cholera porcin.

2. Nulle del secundari invasores bacterial que esseva isolate pareva haber exercite un influentia in le occurrentia del areas de infarcimento. Le sol cognoscite factor commun esseva le presentia de virus de cholera porcin.

3. Le areas de infarcimento in le vesicas biliari pareva esser comparabile con illos observate e reportate in le grande intestino. Illos esseva reguardate como pathognomonic pro cholera porcin.

4. A parte le facto que areas de necrosis focal esseva presente in le vesicas biliari de porcos experimentalmente inficite con virus de cholera porcin, infarcite areas esseva etiam incontrate in porcos con cholera porcin que habeva essite referite al laboratorio diagnostic.

Application of the Conglutination Complement Absorption Test to Detect Hog Cholera Antibodies. I. The Technique

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WHILE serologic tests are available for the more exotic diseases of swine (vesicular exanthema,[1, 19] vesicular stomatitis, [1, 3, 19, 20] and foot-and-mouth disease),[18] a serologic test for hog cholera (HC) is still lacking although the disease was clinically recognized as early as 1833. In addition, attempts to infect laboratory animals (*i.e.*, mice, rats, guinea pigs, and hamsters) have been unsuccessful.[15]

The lack of a test system has denied the veterinarian a practical and accurate diagnostic test for hog cholera. Furthermore, it has prevented the standardization of hog cholera antibodies in anti-hog cholera serum and virus particles in virulent virus, modified virus, or killed virus, all of which are employed either singly or in combination with antiserum to produce immunity to the disease in swine.

The conglutination complement absorption test (CCAT), first described by Bordet and Streng[2] and subsequently modified by Hole and Coombs,[14] has found application in studies with glanders,[13] Q fever,[22] brucellosis,[12] and influenza.[21] In all instances, the CCAT was found to be more sensitive than the ordinary hemolytic complement-fixation (HCFT) test in detecting the presence of antibodies in serum. In addition, it was found that certain antiserums which did not fix hemolytic complement would fix horse conglutinating complement.[5, 16, 17]

In view of the successful applications of the CCAT and the difficulties encountered with the HCFT,[4, 6] the CCAT was applied to the hog cholera system.

MATERIALS AND METHODS

Titration and Conglutinin.—Heat-inactivated (56 C. for 30 minutes) bovine serum diluted 1:10 and 1:20 was absorbed with 5% sheep red blood cells (RBC) and added to a series of tubes in 0.05, 0.10, 0.15, 0.20, and 0.25 ml. amounts. Pooled horse complement (1:10 or 1:20) was added to each tube in 0.4 ml. amounts followed by the addition of 0.4 ml. aliquots of washed (3x) 0.25% sheep RBC. NaCl solution (0.87%) was added to bring the total volume of each tube to 2 ml. The tubes were incubated at 37 C. for 30 minutes, centrifuged at 500 to 600 r.p.m. for 5 minutes, shaken, permitted to stand at room temperature for 5 minutes, and the results recorded. The smallest amount of bovine serum affecting complete conglutination (agglutination) of RBC was considered to be 1 unit. The bovine serum sample diluted 1:10, which showed complete conglutination in 0.1 ml. or 0.05 ml. amounts, was used as the source of conglutinin. The following controls were included in the test: bovine serum + sheep RBC; horse serum + sheep RBC; saline + sheep RBC. None of the control tubes contained evidence of conglutination. Four units of bovine conglutinin in 0.4 ml. was used in the conglutination complement-absorption test (CCAT). Since conglutinin content varies in bovine serum, several pools of it should be titrated simultaneously.

Titration of Complement.—Pools of horse complement, diluted 1:10 and 1:20, were added in 0.05, 0.10, 0.15, 0.20, and 0.25 ml. amounts to replicate series of tubes; 0.4 ml. aliquots of bovine serum containing 4 units of conglutinin and 0.4 ml. amounts of a 0.25% sheep RBC were then added to each tube, followed by sufficient saline solution to bring the final volume in each tube to 2 ml. The tubes were incubated, centrifuged, and shaken and the conglutination recorded. The smallest amount of pooled horse complement causing complete conglutination of sheep RBC in the presence of 4 units of conglutinin was taken as 1 unit. Three or four units of horse complement in 0.4 ml. was used in the CCAT. Pooled horse complement which effected complete conglutination in 0.1 or 0.05 ml. amounts was used. Appropriate controls were included.

Titration of Complement in the Presence of Antigen.—A modified live virus hog cholera vaccine of rabbit origin* was reconstituted, initially diluted 1:10, and subsequently diluted in twofold steps through 1:320. Varying amounts (0.05, 0.1, 0.15, 0.2 and 0.25 ml.) of complement were added to 0.4 ml. aliquots of the diluted antigen. Sufficient saline solution was then added to each set of tubes to bring the volume to 1.2 ml. The tubes were held at room temperature for 30 minutes, after which time 0.4 ml. of bovine serum containing 4 units of conglutinin and 0.4 ml. of 0.25% sheep RBC were added to each tube. The tubes were then incubated at 37 C. for 30 minutes, centrifuged,

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From the Roswell Park Memorial Institute, Buffalo, N.Y. (Millian), and the University of Nebraska, Lincoln (Englehard).

* Rovac (lot 6282-23, expiration date June 24, 1960), produced by Lederle Laboratories, Pearl River, N.Y.

shaken, and allowed to settle before conglutination was recorded. Results indicated that antigen dilutions up to 1:40 were anticomplementary and that antigen dilutions of 1:80 or more were not. The most concentrated antigen dilution producing no anticomplementary effect was taken as 1 unit. One unit of antigen in 0.4 ml. amounts was utilized in the CCAT.

TABLE 1—Titration of Horse Complement in the Presence of Rabbit Origin Hog Cholera Virus*

Vaccine dilution	Pooled horse complement diluted (1:10)				
	0.05 ml.	0.1 ml.	0.15 ml.	0.20 ml.	0.25 ml.
Undiluted	−	−	−	−	−
1:10	−	−	−	−	−
1:20	−	−	−	−	−
1:40	−	−	−	±	±
1:80	±	±	+	+	+
1:160	+	+	+	+	+
1:320	+	+	+	+	+

+ = Conglutination; − = no conglutination; ± = minimum conglutination.
* Rovac, produced by Lederle Laboratories, Pearl River, N.Y.

The results of the titration of complement in the presence of varying amounts of antigen are presented in Table 1. Complement titrated in the presence of normal rabbit tissue (spleen, liver, and blood diluted 1:80) in the absence of virus was not anticomplementary. The 1:80 dilution of normal rabbit tissue was equivalent in protein content to the rabbit tissue components in the vaccine.

DETERMINATION OF OPTIMAL ANTIGEN DOSE IN PRESENCE OF HYPERIMMUNE SERUMS

Pooled hyperimmune serums* were prepared by vaccinating 3- to 4-week-old pigs, 12 to 15 lb. in weight, with live virus vaccine. The pigs were given booster injections of the vaccine, 3 times at weekly intervals and blood samples were obtained 5 weeks following the initial injection.

Twofold dilutions of hyperimmune serums ranging from 1:4 through 1:1,024 were prepared and added in 0.4 ml. amounts to 0.4 ml. aliquots of antigen diluted in twofold steps from 1:10 through 1:640; 0.4 ml. amounts of horse serum, diluted 1:20 or 1:40 to contain 4 units of complement, were then added to the serum-antigen mixtures. Following an incubation period of 30 minutes at 37 C., 4 units of conglutinin (bovine serum) in 0.4 ml. amounts and 0.4 ml. of a 0.25% sheep RBC suspension were added to each tube. The tubes were then reincubated at 37 C. for 30 minutes, centrifuged, shaken, and results recorded. Appropriate controls were included in the test.

The results of the grid titration may be found in Table 2. Since the anticomplementary activity of the vaccine in dilutions of 1:40 or less (Table 1) precluded their use, a 1:80 dilution was considered the most concentrated antigen dilution usable. The 1:80 dilution reacted with hyperimmune serums diluted as high as 1:256. The more dilute antigen preparations (*i.e.*, 1:160, 1:320, and 1:640) detected less antibody (titers of 1:64, 1:32, and 1:16, respectively). One unit of antigen, therefore, consisted of 0.4 ml. of a 1:80 dilution. When normal rabbit tissue (1:80) was used in lieu of the vaccine, conglutination was observed in all the tubes.

TABLE 2—Determination of the Optimal Antigenic Dose Using Hyperimmune Anti-Hog Cholera Serum

Hyperimmune serum dilutions	Hog cholera vaccine* dilutions							
	Undiluted	1:10	1:20	1:40	1:80	1:160	1:320	1:640
1:4	−	−	−	−	−	−	±	±
1:8	−	−	−	−	−	−	±	±
1:16	−	−	−	−	−	−	−	−
1:32	−	−	−	−	−	−	−	2+
1:64	−	−	−	−	−	−	2+	±
1:128	−	−	−	−	−	2+	2+	2+
1:256	−	−	−	−	−	2+	4+	4+
1:512	−	−	−	−	±	2+	4+	4+
1:1,024	−	−	−	−	4+	4+	4+	4+

4+ = Maximum conglutination; 2+ = moderate conglutination; ± = minimum conglutination; − = no conglutination.
* Rovac, produced by Lederle Laboratories, Pearl River, N.Y.

* Supplied by Dr. George Aajoung and Norman Underdahl of the Department of Animal Pathology at the University of Nebraska, Lincoln.

CONGLUTINATION COMPLEMENT ABSORPTION TEST USING NORMAL, IMMUNE, AND HYPERIMMUNE SERUMS

Three different procedures were used to inactivate the substance or substances normally found in pig serums that interfere with conglutination.[4,6,20] The methods were as follows:

1) Heat inactivation only—serums were held at 56 C. for 30 minutes.

2) Periodate treatment—one volume of serum was mixed with 2 volumes of M/90 solution of potassium periodate and held at 4 C. for 18 hours. Two volumes of a 1% glycerol in saline solution was then added.

3) Trypsin-periodate treatment—4 mg. of trypsin (Difco) 1:250 dissolved in 0.5 ml. of phosphate buffered saline (pH 8.2) was added to 1 ml. of serum. The trypsin and serum mixture was held at 56 C. for 30 minutes, after which time 2 ml. of M/90 potassium periodate solution was added. Following a 30-minute incubation period at room temperature, 2 ml. of a 1% glycerine-saline solution was added to the mixture.

The CCAT.—Serial twofold dilutions of the treated serums of 16 normal, immune, and hyperimmune pigs were prepared in 0.4 ml. amounts. The dilutions of the treated serums were corrected to effect initial dilutions of 1:8; 0.4 ml. of freshly reconstituted vaccine, diluted 1:80, and 4 units of horse complement (1:20) in 0.4 ml. aliquots were then added to each tube. The tubes were incubated at room temperature for 30 to 45 minutes after which time 4 units of bovine conglutinin (1:10) in 0.4 ml. amounts and 0.4 ml. aliquots of a 0.25% sheep RBC suspension were added to each tube. The tubes were reincubated at 37 C. for 30 minutes, centrifuged, shaken, and results recorded. A cloudy suspension of RBC indicated a positive test, aggregates of RBC, a negative test.

The following controls were included in the test: (1) Prevaccination and hyperimmune serums in test dilutions mixed with vaccine; (2) dilutions of prevaccination, postvaccination, and hyperimmune serums mixed with normal pooled rabbit tissues (spleen, liver, and blood) of protein content comparable to that of the vaccine; and (3) saline controls (*i.e.*, vaccine + saline, normal rabbit tissues + saline, antiserums + saline, indicator system components + saline).

TABLE 3—The Conglutination Complement Absorption Test Using Heat-Inactivated Anti-Hog Cholera Serum

Serum No.	Antiserum dilutions					
	1:8	1:16	1:32	1:64	1:128	1:256
9	+	−	−	−	−	+
12	+	+	−	−	−	+
14	+	+	+	±	+	±
16	+	+	··	−	±	±
17	+	+	±	+	−	±
19	+	+	+	+	+	±
20	+	−	−	−	±	±
23	+	+	−	−	−	±
24	+	+	+	−	+	±
25	+	−	−	+	±	±
26	+	+	+	−	±	±
27	+	+	±	−	±	+
28	+	+	−	−	±	+
Hyperimmune	±	−	−	±	±	+
Normal	+	+	+	+	+	+

+ = Conglutination; ± = minimum conglutination; − = no conglutination.

RESULTS AND DISCUSSION

The results of parallel CCAT titrations using heat-inactivated, periodate-treated, and trypsin-periodate treated antiserums were tabulated (Tables 3, 4, and 5). Depletions of serums necessitated the reduction in the number of serums tested in several instances.

The poorest results in terms of total number of serums reacting and serums sensitivity (expressed as titers) were observed in the nontreated heat-inactivated serums. In this group, only 11 of the 13 known positive serums reacted with the antigen and bound complement. Few serums were active at dilutions of 1:64 or higher. In addition, a marked prozone effect was apparent in most of the serums tested. (Dilutions as high as 1:32 were masked by this phenomenon.)

All the 14 positive periodate-treated serums and the 9 trypsin-periodate-treated

TABLE 4—The Conglutination Complement Absorption Test Using Periodate-Treated Anti-Hog Cholera Serum

Serum No.	Antiserum dilutions					
	1:8	1:16	1:32	1:64	1:128	1:256
9	+	−	−	−	−	±
12	−	−	−	−	−	±
14	−	−	−	−	−	±
16	−	−	−	−	±	±
17	±	−	−	−	−	−
19	±	−	−	−	−	±
20	±	−	−	−	±	±
23	+	−	−	−	−	±
24	−	−	−	−	−	±
25	+	−	−	−	±	±
26	+	−	−	−	−	±
27	±	−	−	−	−	±
28	−	−	−	−	−	±
30	+	+	−	−	−	−
Hyperimmune	±	−	−	−	−	−
Normal	+	+	+	+	+	+

+ = Conglutination; ± = minimum conglutination; − = no conglutination.

TABLE 5—The Conglutination Complement Absorption Test Using Trypsin-Periodate-Treated Anti-Hog Cholera Serum

Serum No.	Antiserum dilutions					
	1:8	1:16	1:32	1:64	1:128	1:256
9	±	−	−	−	−	+
12	−	−	−	−	−	±
14	−	−	−	−	−	±
16	−	−	−	−	±	±
17	−	−	−	−	−	±
19	±	−	−	−	−	±
24	±	−	−	−	−	±
26	+	−	−	−	−	±
30	+	−	−	−	−	±
Hyperimmune	±	−	−	−	−	−
Normal	+	+	+	+	+	+

+ = Conglutination; ± = minimum conglutination; − = no conglutination.

serums reacted with the antigen and bound complement. The sensitivities (titers) of the 2 groups of treated serums were comparable. Three of the periodate-treated serums titered 1:64, 9 titered 1:128, and 2 titered 1:256. One of the trypsin-periodate-treated serums titered 1:64 and 8 titered 1:128. Prozone reactions were observed at dilutions of 1:8 in 9 periodate-treated serums and in 5 of the trypsin-periodate-treated serums.

Prevaccination and normal control serums did not react with the vaccine and bind complement, as maximal conglutination was observed in these series. Hyperimmune serums did, however, bind complement in a dilution as high as 1:256.

In no instance did any of the prevaccination, postvaccination, normal control, or hyperimmune serums react with the normal rabbit tissue control and bind complement. Conglutination was also evident in all saline controls in which the complete indicator system was present.

Preliminary studies using commercial HC antiserums and virus in the CCAT indicated comparable sensitivities.

These data indicate that the CCAT may be used to detect conglutinating antibodies formed as a consequence of immunization. Utilizing this procedure, it is now possible to quantitate the potencies of antiserum preparations *in vitro* and thereby establish minimal standards for commercial anti-hog cholera serum preparations. The CCAT also should enable one to determine the efficacies of the available commercial hog cholera vaccines without resorting to challenging the immunity of pigs with graded doses of virulent virus. Furthermore, one may now accurately determine the best time to kill immune hogs for maximal antibody yields —a necessary prerequisite for economic commercial antibody production.

By using standard hyperimmune serums it should also be possible to quantitate and standardize commercial HC voccines. The test may also clarify the "variant problem."[7-11]

The data also indicate that pretreatment of serums with either periodate or trypsin-periodate facilitates antibody detection. While the nature and characteristics of the inhibitor[s] present in both normal and immune porcine serums[4,6] have not been elucidated, it is apparent that its effect must be mediated to effect a valid, reproducible titration. Treatment of antiserums by periodate and by trypsin-periodate have proved to be effective in reducing and even eliminating the effect of nonspecific inhibitors in other serologic systems.

Some preliminary insight as to the nature of the inhibitor[s] may, however, be gleaned from the pretreatment data. The results suggest that the inhibitor[s] is heat stable but may be oxidized. Further characterization of the inhibitor[s] in porcine serums should prove interesting.

SUMMARY

The conglutinin complement absorption test (CCAT) may be used to detect hog cholera antibodies. The results of parallel CCAT titrations using heat-inactivated, periodate- and trypsin-periodate-treated antiserums are presented. The poorest results in terms of number of serums reacting and serum sensitivity (expressed as titers) were observed in the heat-inactivated group of serums. Data indicate that pretreatment of serums with either periodate or trypsin-periodate facilitates antibody detection. The sensitivities of these treated groups were comparable (titers as high as 1:256 were observed).

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SUMMARIO IN INTERLINGUA

Application del Test de Absorption de Complemento de Conglutination al Detection de Anticorpore Anti Cholera Porcin
I. Le Technica

Le test de absorption de complemento de conglutination (TACC) pote esser usate pro deteger anticorpore anti cholera porcin. Le resultatos es presentate de titrationes de TACC effectuate parallelmente con le uso de antiseros (1) thermo-inactivate, (2) tractate con periodato, e (3) tractate con trypsina e periodato. Le resultatos le minus satisfacente quanto al numero del seros reagente e al grado de sensibilitate (exprimite in le titros) esseva observate in le thermo-inactivate gruppo de seros. Le datos indica que le pretractamento del seros con periodato o con periodato e trypsina promove le detection de anticorpore. Le sensibilitates observate in le duo tractate gruppos esseva comparabile. Le titros incontrate includeva 1:256 como maximo.

Toxicity of Wood Preservatives for Swine

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VERTICAL INTEGRATION of the swine industry has brought about extensive changes in management procedures. Attempts to bring about greater utilization of space in farrowing houses has created an increasing interest in the farrowing crate. Some crates have removable wooden platforms which are exposed constantly to the secretions of the sow during use. Wood preservatives are often utilized to extend the life of these wooden platforms.

Investigation of extensive losses of baby pigs in the immediate area of this laboratory indicated the possibility that a wood preservative toxicity existed.[8] Sows that farrowed in the same farrowing house but on floors or platforms untreated with wood preservatives delivered generally healthy litters. Platforms, treated with wood preservatives and observed to be toxic to baby pigs, were covered with ¼-inch plywood for subsequent farrowing. Sows farrowing on the plywood-covered platforms delivered and raised healthy pigs.

Toxicity of pentachlorophenol (PCP) for dogs, rabbits, rats, and guinea pigs has been demonstrated.[2, 3]

Walters [10] demonstrated that PCP was not toxic to swine when applied to wooden feed and water troughs and farrowing houses; the baby pigs were brought into contact with the treated lumber in the farrowing house after they were 2 weeks old. Drenching 3-month-old pigs with a solution containing 30 Gm. of PCP did not produce death, but some microscopic lesions were found in the liver, spleen, and kidneys. The dosage of PCP administrated orally was presumed much greater than the intake by swine in contact with lumber to which PCP solutions had been applied. Bohosiewicz [2] described the clinical signs in foals, dogs, young cattle, and fowls exposed to weed-killers, insecticides, and wood preservatives of the dinitrophenol and dinitro-ortho-cresol types.

Harrison[5] demonstrated possible fatal chronic dose rates for swine to be in the range of 27 to 55 mg./kg. of body weight and presented clinical, postmortem, and analytical data for PCP poisoning. It was thought that creosote was more toxic than PCP to calves and that wood containing creosote preservatives presented no hazard to livestock if correctly utilized. Other investigators have demonstrated toxicity of PCP and creosote under various conditions.[1, 4, 6, 7]

EXPERIMENTAL PROCEDURES AND RESULTS

All sows utilized in this investigation farrowed in individual, all-wood houses. Two weeks previous to farrowing, each sow was given 120 ml. each of anti-hog cholera serum and anti-erysipelas serum. Immediately following completion of farrowing, each sow was given 1 Gm. intramuscular oxytetracycline administered deep into the thigh muscles to aid in the prevention of enteritis.[9] All except 2 sows farrowed in farrowing crates with wooden platforms that had been treated with various wood preservatives and their carrier vehicles. The other 2 sows farrowed in a farrowing house in which the floor was constructed of fir lumber treated with PCP by commercial process. Straw bedding was placed on the floor previous to and following farrowing. No bedding was utilized on the platforms of the farrowing crates.

Iron dextran (96 mg.) was given intramuscularly to all live pigs 5 days following farrowing.

EXPERIMENT 1

Eight sows farrowed in wooden farrowing crates with wood (fir) platforms that had been treated at least 3 times with the fol-

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lowing commercial PCP wood preservative preparation, designated as dilute PCP:

	%
Pentachlorophenol	4.37
Other chlorophenols	0.63
Petroleum distillate	81.60
Inert ingredients	13.20

All applications were made with a brush, and the platforms were allowed to air-dry for at least 24 hours between each application. The platforms dried for 1 to 5 days before confinement of the sow. The sows were confined to the farrowing crates 1 to 12 days previous to farrowing.

The 8 sows delivered 89 pigs, 5 of which were dead at birth. The rate of mortality at birth was greatest for those sows confined for longer periods before farrowing. Typical lesions of PCP toxicosis were observed in 15 pigs that died following farrowing. Most of the surviving pigs were found to have various signs of contact with toxic materials. Necrosed areas of varying degree and size were observed on the mammary glands of all sows.

EXPERIMENT 2

Two days prior to farrowing, 3 sows were confined to farrowing crates with wooden platforms constructed of fir lumber factory-treated with PCP. The sows delivered 33 pigs, 5 of which were dead upon delivery. Ten of the pigs died within 6 days following delivery, and the various lesions of PCP toxicosis were observed. One sow was removed from the farrowing crate 5 days following farrowing and a replacement was made. The replacement sow farrowed 3 days following confinement, delivering 8 pigs, 3 of which were dead. No further losses were recorded.

EXPERIMENT 3

Two sows were confined to farrowing crates with wooden (pine) platforms to which undiluted PCP had been applied by brush. Three applications were made and the platforms were air-dried. The sows were confined 3 days after the last application. Both sows developed signs of irritation within 5 hours following confinement. Both sows died within 24 hours following confinement, having extensive abdominal burns and necrosis. Upon postmortem examination, swollen, edematous lymph nodes throughout the entire body, extensive pneumonic lesions, and acute emphysema were seen, as well as extensive hyperemia and necrosis of the skin of the mammary glands. A peracute necrotic mammitis was also observed.

No pigs were delivered by either sow.

EXPERIMENT 4

Five days prior to farrowing, 2 sows were confined to a farrowing house, the floor of which was made of fir lumber factory-treated with PCP. Wheat straw bedding was used previous to and following farrowing. Nineteen pigs were delivered, with 1 dying on the 1st day following farrowing. The cause of death was undetermined. No additional losses were recorded.

EXPERIMENT 5

Four sows farrowed in farrowing crates with wood (fir) platforms that had been given at least 3 brush applications of a commercial wood preservative preparation consisting of 98.5% coal tar creosote and 1.5% inert ingredients. The treated platforms were allowed to dry for 24 to 48 hours before confinement of the sows. The sows were confined to the farrowing crates 2 and 10 days previous to farrowing.

The 4 sows delivered 41 pigs, 24 of which were dead on delivery. The surviving pigs were rough and dehydrated, having rough skins and severe diarrhea.

EXPERIMENT 6

One sow was confined to a farrowing crate with a wooden (pine) platform to which 3 brush applications of distillate were made. The sow was confined for 3 days previous to farrowing. Eleven pigs were delivered, all of which were alive. There were no post-farrowing losses.

EXPERIMENT 7

Four sows were confined to farrowing crates with wood platforms made of untreated pine lumber. Confinement was established at least 24 hours before farrowing. Thirty-six pigs were delivered with 3 deaths within 24 hours following delivery. Death was believed to be due to crushing. No further losses were recorded.

A summary of these first 7 investigations is presented (Table 1).

EXPERIMENT 8

Two pigs, weighing 25 and 30 lb., were given orally 15 and 30 ml., respectively, of

TABLE 1—Mortality of Pigs Farrowed in Crates, the Wooden Platforms of Which Had Been Treated with Wood Preservatives or Carrier Vehicles

Treatment *	Experiment No.	No. of pigs in experiment**	No. dead on delivery	Post-farrowing deaths†
Dilute PCP‡	1	8/89	5	1/10 2/4 3/1
Dilute PCP (factory-treated lumber)	2	3/33	5	1/4 3/4 4/1 6/1
Undiluted PCP	3	2/0	Both sows died before delivery	
Dilute PCP (factory-treated lumber covered with straw)	4	2/19	0	1/1
Undiluted creosote	5	4/41	24	1/6 2/4 3/1
Distillate	6	1/11	0	0/0
Controls	7	4/36	1	1/1 2/2

* Applied by brush unless otherwise described.
** Numerator = number of litters investigated; denominator = number of pigs in litters.
† Numerator = number of days following delivery; denominator = number of pigs dying.
‡ PCP = pentachlorophenol.

the PCP solution described in experiment 1. Signs of abdominal pain were evident immediately following administration. Extensive retching and vomiting were observed within 1 minute following administration. Both pigs had definite signs of depression and posterior paralysis within 5 minutes following administration. Both consumed large quantities of water for the 1st half hour, and much of it was regurgitated. Signs of toxicosis were not observed after 8 hours or during a 3-week observation period.

Clinical Signs

Pigs farrowed by sows having abdominal contact with lumber treated with wood preservatives were reluctant to nurse. Signs of starvation were evident. Various degrees of dehydration and depression were usually observed in these baby pigs. Burns on the nostrils, tongue, and other parts of the face were also seen (Fig. 1, 2). In some instances, watery diarrhea was evident. Sows had necrosis of the skin of teats and mammary glands if they were confined for 5 days or more.

Pigs dead upon delivery were usually enclosed within their fetal membranes. Suffocation was considered 1 of the causes of death. Necropsy lesions were usually less severe but similar to those lesions observed in the pigs dying after delivery.

Pathologic Changes in Baby Pigs

Variations of degree and type of lesions existed between pigs of the experimental groups, but were usually quite constant within each experimental group. The most often observed lesions are described.

Kidney.—Lesions included subcapsular fluid and petechial to ecchymotic hemorrhages. A spongy texture was usually observed.

Urinary Bladder.—The fluid present within the bladder was white and tenacious and contained fibrinous threads.

Liver.—The livers were congested and

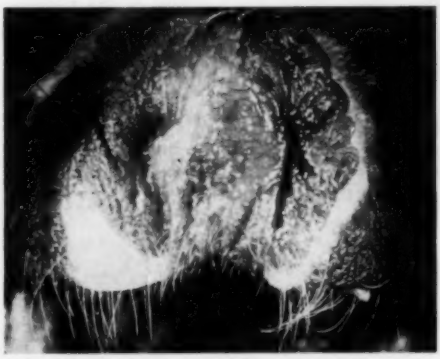

Fig. 1—Necrotic burns on the nostrils of a baby pig.

Fig. 2—Burns and necrotic areas on the tongue of a suckling pig.

dark chocolate in color. Some infarcts were observed on the borders.

Spleen.—Some spleens were congested and there were infarcts of various sizes along the borders.

Stomach.—Catarrhal inflammation and petechial hemorrhages were consistent lesions of the mucosal surface. The serosal surface was consistently pale, with infrequent ecchymotic hemorrhages. The presence of extensive gas was consistently observed.

Intestinal Tract.—The mesentery was severely congested with enlarged, dark, watery, lymph nodes in all cases. There was generalized catarrhal inflammation in the small intestine. An extensive amount of gas was usually present in the large intestine.

Respiratory Tract.—The external nares had various degrees of necrosis. Mild emphysema and congestion were observed in the lungs, with some areas of consolidation.

Oral Cavity.—Various degrees of coagulation necrosis on the borders of the tongue and lips were detected in all instances.

Some pigs appeared to have been crushed, but various degrees of the lesions described were found at necropsy. Close observation indicated that the pigs affected by the toxic action of PCP or creosote had difficulty in moving and thus were more subject to crushing than healthy pigs.

DISCUSSION

Investigations utilizing pregnant swine indicated that direct contact with lumber freshly treated with solutions of PCP or creosote would produce acute toxicosis, resulting in extensive mortality in newborn swine.

Lumber extensively saturated with wood preservatives and insufficiently dried appeared to be the most toxic to swine.

The direct contact of the pregnant sow with lumber freshly treated with PCP or creosote apparently provided sufficient skin absorption to cause fetal deaths and weak pigs at birth. Pigs delivered dead were usually encased in fetal membranes. Direct contact of the lactating sow with freshly treated lumber discouraged nursing due to the presence of PCP or creosote on the teats and mammary glands. When nursing did occur, toxic quantities of PCP or creosote were apparently obtained orally by the pigs. The terminal result in either case was starvation and extensive mortality due to absorbed quantities of toxic materials.

The presence of the sow and her daily secretions, with the concomitant exposure to the atmosphere and routine cleaning procedures, aid in elimination of excessive toxic materials present in wood treated with preservatives. The body heat of the sow apparently aids in the expulsion of excessive wood preservatives in freshly treated lumber. Decreased toxicity was observed for sows and their litters when farrowing occurred on treated platforms which had been utilized previously for one farrowing.

When bedding was applied to newly treated floors, toxicosis was not observed in the sow or her litter. Mortality was greatest in litters from sows confined to freshly treated plaforms for long periods before farrowing.

The weight gains of pigs were hindered by PCP or creosote until the pigs reached approximately 5 to 6 weeks of age (Table 2). Pigs 1 week old or older appeared to possess a greater tolerance to PCP or creosote. A possible explanation is that their metabolism had progressed to the point that elimination through the feces and urine was more rapid and complete.

TABLE 2—Comparison of Average Weights (lb.) of Experimental Pigs Involved in Experiments Designed to Determine Toxicity of Wood Preservatives

Preservative	Total No. pigs	Age (days)			
		Birth	7	21	42
Pentachlorophenol	141	3.4	6.1	12.3	24.2
Creosote	41	2.9	4.3	10.0	20.8
Distillate	11	3.0	8.6	21.1	27.4
Controls	36	3.17	8.28	20.68	25.3

Contact with undiluted PCP was irritating to experimental sows. Extreme nervousness and excitement ensued within 5 hours after contact and terminated in death.

No toxicosis was observed in pigs having contact with distillate-treated platforms. Distillate was studied because it is often utilized as a diluent for PCP.

The application of PCP, creosote, or distillate to pine lumber provided a greater and more complete absorption than when applied to fir lumber. Lumber used in this investigation was purchased from lumber yards. It had been commercially treated with PCP and appeared dry on the surface. However, when placed in a heated room or in direct exposure to the heat of the sun,

the chemical could be detected exuding from the lumber. The exuding process was more evident for fir lumber than for pine lumber and was particularly evident in areas surrounding knots.

SUMMARY

Wood preservatives containing pentachlorophenol (PCP) or creosote may be extremely toxic to young swine coming into direct contact with them. The degree of toxicity lessens as the pigs become older.

Toxicosis is particularly evident in swine having extended direct contact with freshly treated lumber containing excessive quantities of wood preservatives. Liberal amounts of bedding aid in preventing the condition.

Wood preservatives containing PCP or creosote, when properly applied to wood which is thoroughly dried, appear to have little or no toxicity for swine.

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SUMMARIO IN INTERLINGUA

Toxicitate de Preservativos de Ligno in Porcos

Preservativos de ligno que contine pentachlorophenol (PCP) o creosoto pote provar se extrememente toxic pro juvene porcos exponite al contacto con illos. Le grado del toxicitate declina in tanto que le porcos deveni plus matur.

Toxicosis es particularmente evidente in porcos exponite durante prolongate periodos al contacto directe con frescamente tractate productos de lignos que contine excessive quantitates de preservativo. Le provision de abundante quantitates de material de lectiera es de valor in le prevention del disordine.

Preservativos de ligno que contine PCP o creosoto e que es applicate appropriatemente a ben seccate lignos pare haber pauc o nulle toxicitate pro porcos.

Effects of Prenatal *Gamma* Radiation on Testicular Morphology and Function in Swine

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A COMPREHENSIVE review of the literature concerning the effects of prenatal radiation on mammalian development has been published by Russell.[14] A few of the reports included in her review mention testicular atrophy and impaired fertility in mice or rats. Rugh and Jackson[13] reported reduced fertility in male mice following 200 r x-irradiation on days 15.5 and 16.5 of gestation. Ershoff[3] reported severe testicular damage in male rats irradiated on the 18th day of gestation. Little additional work on the effects of prenatal radiation on reproductive performance has been reported. The following study was conducted to determine the effects of a single sublethal exposure of gilts in midgestation to *gamma* radiation on the viability, growth, testis morphology, and semen characteristics in the male offspring.

MATERIALS AND METHODS

The boars used in this study were produced by 16 crossbred Hampshire-Yorkshire gilts bred to a Duroc boar. During the breeding period the gilts were inspected for estrus twice daily by means of a testosterone-treated barrow. Each gilt was served twice by a boar, the first near the beginning of estrus and the second 8 to 12 hours later.

On Jan. 15, 1958, 8 gilts 54 to 64 days pregnant were simultaneously exposed (total body) to an air dose of 400 r *gamma* radiation from a multicurie cobalt-60 source (Wilding *et al.*,[18] Trum *et al.*[16]) at a rate of 24 r per hour as calculated by Victoreen dosimeters.

Parturition occurred in farrowing crates. The number of pigs, sex, and individual birth weights were recorded for each litter. Weights were obtained at 28 and 56 days of age. All pigs were given 5-ml. oral doses of a saturated ferrous sulfate solution at weekly intervals until moved to pasture at 4 weeks of age. At 56 days of age, the male pigs were returned to the barn and maintained in pens with concrete floors for the duration of the experiment. Due to limited facilities, all female pigs were killed at weaning. The self-fed rations were formulated to meet NRC recommendations[9] for pregnant or lactating gilts and growing pigs. Control (C) and irradiated (I) boars were penned together until about 8 months old.

Fifteen C and 20 I boars were castrated at 120 days of age to obtain data on testes weights and morphology. The remaining boars in both groups (28 C and 10 I) were given several opportunities to serve an estrous gilt restrained in a breeding crate. Subsequently, attempts were made to have each boar serve an artificial vagina mounted in a phantom sow. Eight C and 3 I boars, which would not serve the artificial vagina, were castrated at about 204 days of age; 5 C and 7 I boars were castrated at 254 days of age.

Each testis was weighed and a wedge-shaped slice fixed in Lavdowsky's mixture of formalin, alcohol, and acetic acid (Guyer[6]). Sections 7 μ thick were stained by the periodic acid-Schiff technique (Hotchkiss[7]).

The cross-sectional diameters of seminiferous tubules from each boar in each age group were measured after projection onto a screen with a Leitz microprojector. The largest and smallest diameters of 30 tubules from each boar were averaged to obtain the mean diameter of the seminiferous tubules.

Three hundred seminiferous tubules from each boar in the 254-day group were microscopically examined and classified as active or inactive. A tubule was considered active if it contained spermatogonia even though the cells were few and unevenly spaced along the basement membrane.

Semen was collected from 5 C and 5 I boars; however, 1 of the I boars produced so few sperm the semen could not be satisfactorily analyzed. The C boars were randomly selected from 15 which had been trained to serve an artificial vagina. The 5 I boars were the only ones from a group of 10 that consistently served the artificial vagina. Semen was collected on Monday and Friday for 3 successive weeks, followed by daily collections on Monday through Saturday and on Monday and Tuesday of the following week. These boars were 225 to 240 days of age at the beginning of this phase.

The semen was evaluated on the basis of the following criteria: (1) strained semen volume, (2) sperm concentration, (3) rate of motility and

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percentage of motile sperm, (4) percentage of live sperm, (5) percentage of abnormal sperm, (6) initial fructose concentration in semen, and (7) oxygen utilization during a 75-minute incubation period.

After straining through cheesecloth to remove the tapioca-like fraction of the ejaculum, the semen volume was recorded. Sperm concentration was determined with a Cenco photelometer immediately after collection of the semen. Standard curves were constructed by comparing sperm counts in a hemocytometer with the percentage light transmission in the photelometer of 1:4, 1:9, 1:19 dilutions of the semen in a 2.8% solution of sodium citrate. The correlation coefficients between hemocytometer counts and the log of percentage light transmission for the 3 dilution rates were −0.93, −0.96, and −0.95, respectively. The percentage light transmission values for the 1:9 dilutions were used when these values were between 20 and 50; otherwise, the higher or lower dilution as dictated by the initial reading of the 1:9 dilution was used.

Sperm motility was estimated microscopically by placing a drop of undiluted semen under a coverslip on a warm slide. For estimation of rate of motility, the following scale was used: 0 = no motility, 1 = fair, 2 = good, 3 = very good, and 4 = excellent. The percentage of motile sperm was estimated from 0 to 100 in increments of 10.

Estimates of the percentage live and percentage abnormal sperm were obtained from fast green-eosin-stained slides (Mayer *et al.*[6]). Two hundred sperm from each ejaculum were counted and the percentage of unstained sperm recorded as percentage live sperm. Two hundred sperm from the same slide were classified as normal or abnormal in form.

Oxygen utilization under aerobic conditions was determined by the direct method of Warburg.[17] Semen, 2 ml., was placed in a 7-ml. flask and 0.2 ml. of a 10% solution of potassium hydroxide placed in the center well. Air was used for the gas phase. After a 10-minute equilibration period, oxygen utilization at 15-minute intervals during a 75-minute incubation period was determined. The data for each 15-minute period and for the entire 75-minute period were expressed as microliters of oxygen used per hour.

The initial fructose concentration in the semen was determined by the resorcinol reaction (Roe).[12] The semen was prepared for fructose analysis by a method similar to that of Freund *et al.*[5] Only the semen collected at 3- or 4-day intervals was used for fructose concentration and O_2 utilization studies.

RESULTS

Birth and Growth Data.—All 16 gilts farrowed. The 8 C gilts produced 95 pigs (50 males and 45 females) and the 8 I gilts produced 88 pigs (40 males and 48 females). Analyses of the data failed to reveal differences between C and I litters in sex ratio, in litter size, viability, birth weights, and growth rates to weaning age (Table 1). No defects were observed except in 2 I litters (1 irradiated the 59th day and the other the 60th day after breeding) in which the eyelids did not separate until some hours after birth.

TABLE 1—Litter Size Growth Rate and Viability of Control and Irradiated Pigs

Items	Groups	
	Control	Irradiated
No. litters	8	8
Litter size*	11.9 ± 2.5	11.0 ± 2.6
Birth weights (lb.)*	2.8 ± 0.7	2.6 ± 0.6
Weaning weights (lb.)*	30.0 ± 6.0	31.0 ± 6.0
Born alive (%)	96	95
Weaned (%)	87	84

* Means ± standard deviations.

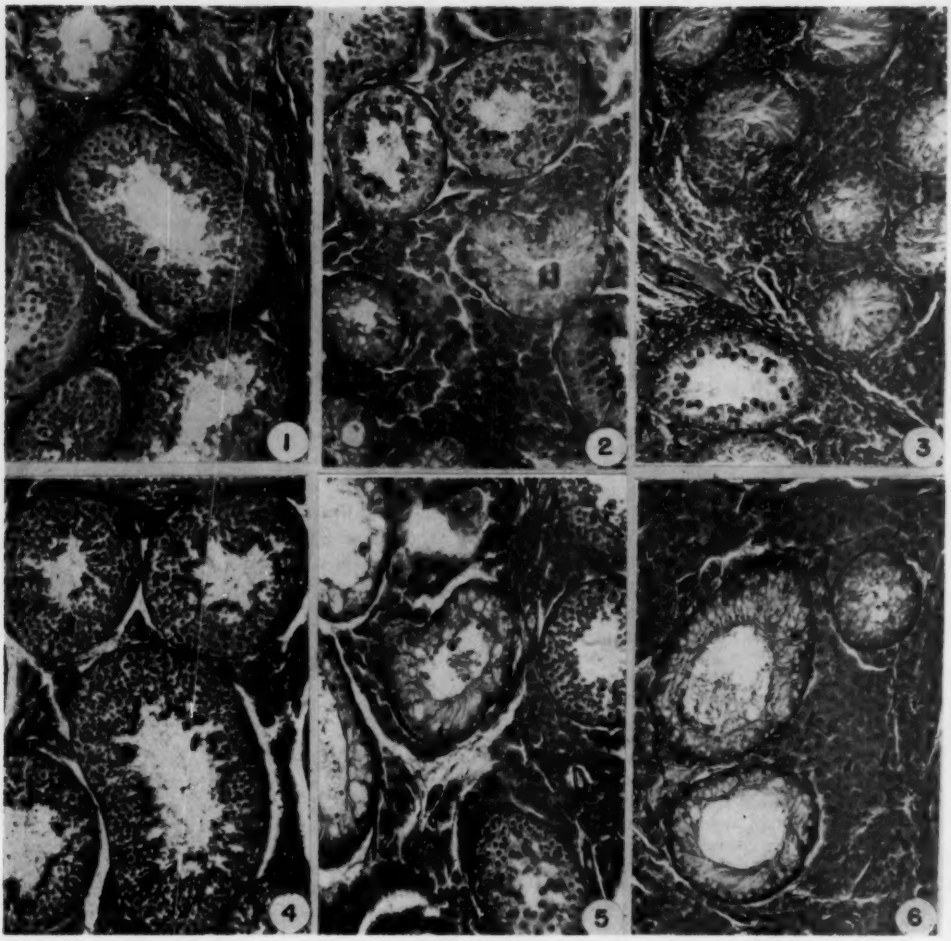
Testicular Development.—The average testes weights of the I boars castrated at 120, 204, or 254 days of age were all less than the testes weights of comparable age control boars (Table 2). The percentage difference between testes weights remained essentially the same (72, 76, and 73% of controls at 120, 204, and 254 days, respectively). Body weights of the C and I groups at comparable ages did not differ ($P > .05$).

TABLE 2—Weights and Functional Development of Porcine Testes

Items	Age at castration (days)		
	120	204	254
Testes wt. (Gm.)			
Control	86 ± 60 (15)	532 ± 195 (8)	720 ± 93 (5)
Irradiated	62 ± 24 (20)	403 ± 55 (3)	528 ± 112* (7)
Diameter of seminiferous tubules (μ)			
Control	124 ± 37	.....	243 ± 25
Irradiated	103 ± 20†	.....	221 ± 67†
Active seminiferous tubules (%)			
Control	.....	.....	100
Irradiated	.....	.....	68†

Number of animals from which the data are taken is shown in parentheses. * $P < 0.05$. † $P < 0.01$.

Examination of the histologic sections of the testes of the I boars revealed wide variation in degree of activity in the seminiferous tubules (Fig. 1–6). The tubules of some I boars appeared normal, while in others the damage varied from slight to almost complete absence of germinal cells (Fig. 2, 3, 5, and 6). Microscopic observations suggested an increase in the total quantity of intertubular tissue; however, no quantitative measurements were made.



Photomicrographs from preparations fixed in FAA and stained with periodic acid-Schiff reaction.

Fig. 1—All stages of spermatogenesis can be seen in this section of testis from a 122-day-old control boar.

Fig. 2—Retardation of spermatogenesis and smaller size of some seminiferous tubules can be seen in this section of testis from a 118-day-old boar (mother exposed to 400 r gamma irradiation on 60th day of gestation); litter mate to boar in Figure 5.

Fig. 3—In this section of testis from 117-day-old irradiated boar (mother exposed to 400 r on 59th day of gestation), there are few tubules with active spermatogenesis; litter mate to boar in Figure 6.

Fig. 4—Section of testis from 247-day-old control boar.

Fig. 5—Section of testis from 247-day-old irradiated boar; normal and atrophic seminiferous tubules appear immediately adjacent to each other. Approximately 30% of tubules in irradiated boars were similar in appearance to the atrophic tubules shown in left side of this section.

Fig. 6—Section of testis from 252-day-old irradiated boar; only 2% of the seminiferous tubules contained evidence of spermatogenesis. Notice apparent increase in quantity of interstitial tissue in sections from all irradiated animals.

TABLE 3—Semen Characteristics of Control and Irradiated Boars

Items	Frequency of semen collections*			
	Twice weekly		Daily	
	Control	Irrad.	Control	Irrad.
Semen volume (ml.)	99 ± 35**	78 ± 32†	83 ± 36	64 ± 26†
Sperm concentration (x 10^6 per ml.)	240 ± 74	202 ± 82	128 ± 81	139 ± 96
Sperm per ejaculum (x 10^8)	234 ± 78	141 ± 57†	108 ± 84	76 ± 48
Fructose per ml. of semen (mg.)	0.44 ± 0.28	0.46 ± 0.17	.....	.....

* A minimum of 20 ejaculums are represented in each of the values listed. ** Mean ± standard deviation. † $P < 0.01$.

The average diameters of the seminiferous tubules of the 3 groups of irradiated boars were less than the tubule diameters of the comparable age control boars ($P < .01$). Due to the small number of irradiated boars in the 204-day group, only the data for the 120- and 254-day age groups are shown in Table 2. The difference in tubule size was due to the smaller size of the inactive tubules in the I boars. The average diameter of the inactive tubules was 183 μ as compared with 243 and 246 μ for the active tubules in control and irradiated boars respectively, at 254 days of age.

None of the seminiferous tubules in the control boars were called inactive, whereas an average of 30% of the tubules in the irradiated boars were called inactive (Table 2). In addition, some of the tubules classified as active in the I boars may not have had a normal sperm-producing potential since they appeared to contain fewer germinal cells than the control tubules.

Semen Production.—The average semen volume, sperm concentration, and total sperm per ejaculum for 5 C and 4 I boars are shown in Table 3. There was no difference ($P > .05$) between the 2 groups in sperm concentration. However, the I boars produced approximately 22% less semen and 35% less sperm per ejaculum than the C boars ($P < .01$).

There were no differences in sperm motility, percentage live sperm, or abnormal sperm in the semen from the 2 groups of boars. The average initial fructose concentration in the semen of the C and I boars was 0.44 and 0.46 mg./ml., respectively.

Sperm concentration of the I boar (not included in the I group, Table 3), was so low that it could not be read in the photelometer. Hemocytometer counts made on 7 ejaculums showed an average of 12 million sperm per milliliter as compared with 139 million per milliliter for the other 4 I boars. In the histologic sections from the testes of this boar, 98% of the seminiferous tubules were inactive (Fig. 6). His average initial fructose concentration was 0.78 mg./ml.

There was no difference between the semen of the 2 groups in total oxygen utilization during the 75-minute incubation period when calculated either on the basis of per milliliter of semen or per billion sperm (Table 4). However, when the data for each group were examined by 15-minute periods, the C boar sperm had a higher initial oxygen utilization and a progressive decline in rate of utilization as compared with a lower initial utilization and no change in rate of utilization by the sperm of the I boars. Correlation analyses indicated that a portion of the decline in rate of oxygen utilization in the C boars may have been due to a more rapid depletion of the available fructose in the semen.

TABLE 4—Oxygen Utilization by Semen of Control and Irradiated Boars

	No. semen samples	Minutes					
		0–75	0–15	16–30	31–45	46–60	61–75
		μ liters of O_2 utilized per hour					
1 ml. of semen							
Control	31	42	48	46	43	38	35
Irrad.	25	40	39	40	44	38	38
1 billion sperm							
Control	31	201	248	225	201	174	157
Irrad.	25	204	200	214	226	188	192

Values underscored by the same line are not significantly different from each other.

DISCUSSION

A number of investigators have observed anomalous development of the eyes, teeth, feet, tails, and internal organs in mice and rats irradiated during the period of organogenesis. Conversely, irradiation during the fetal period seldom produces morphologically defective animals (Russell [14]). Thus it is not surprising that no deformed pigs were produced since they had passed the period of organogenesis at the time of irradiation and were well into the fetal period (Patten [11]).

As mentioned, scanning of the histologic sections gave the impression of an increased amount of intertubular tissue in the testes of some of the irradiated boars. However, this increase may be only relative as reported by Chalkley,[1] Eschenbrenner *et al.*,[4] and Sargent and McDonald.[15] These authors reported that a decrease in intratubular tissues resulted in a relative increase in interstitial tissue but this was not confirmed by quantitative analysis.

The reduction in total sperm production in the irradiated boars was roughly proportional to the reduction in percentage of active seminiferous tubules. That there was no difference in the quantity of oxygen utilized by the sperm of the C and I boars during the 75-minute incubation period indicates no major effect on the metabolic activity of the sperm from the irradiated males. The reduction in semen volume is probably due to the reduction in size of the accessory glands in the irradiated boars; unpublished data in this laboratory show a reduction of approximately 17 and 26% in mature prostate and seminal vesicle weights, respectively, in rats irradiated on the 17th day of gestation. Furthermore, these data contain no evidence of a deleterious effect on mature testes weights or morphology when irradiated prior to the 17th day of gestation; however, irradiation on the 17th or 18th through the 22nd day of gestation led to a reduction of 40 and 80%, respectively, in testes weights as compared with control testes weights. The testes of the rats irradiated on the 17th prenatal day were similar in histologic appearance to the boar testes in this study.

The critical periods of susceptibility to radiation damage for different organ systems in laboratory animals as described by Russell [14] may not be as sharply delineated in animals with longer gestation periods. This question cannot be satisfactorily answered until the data from groups of animals of several species irradiated at all stages of gestation are available. Otis and Brent [10] prepared a table of equivalent ages of nonirradiated mouse and human embryos by matching stages of embryonic structures in both organims. Integration of similar data from several species, including man, with data on the effects of radiation at comparable development stages in laboratory and farm animals will permit more valid extrapolation of the knowledge of radiation effects in lower animals to man.

No tissue-dose measurements were obtained, thus there is no good estimate of the level of radiation received by the fetal testes. Position of the gilts in relation to the radiation sources, thickness of maternal tissues, etc., could have influenced the amount of radiation received by the fetuses, both within and among litters (Trum *et al.*[16]). Gonad-dose measurements are essential if species comparisons are to be meaningful.

SUMMARY

Data on testes weights, testis morphology, and semen characteristics of boars exposed to *gamma* radiation (400 r from Co^{60} given to their dams) between the 54th and 64th day of gestation were obtained. Testes weights of irradiated males were approximately 30% less than those of comparable age control males. There was no difference in the average sperm concentration; however, the irradiated boars produced approximately 22% less semen and 35% less sperm per ejaculum than the control boars. No difference in initial fructose concentration in the semen or oxygen utilization during a 75-minute incubation period was observed.

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SUMMARIO IN INTERLINGUA

Effectos de Irradiation Gamma Prenatal Super le Morphologia e le Function Testicular in Porcos

Esseva obtenite datos relative al peso del testes, al morphologia testicular, e al characteristicas del semine de porcos mascule exponite in utero a irradiation gamma (administrate in doses de 400 r ab Co^{60} a lor matres) inter le 54te e le 64te die del gestation. Le pesos del testes in irradiate masculos esseva approximativemente 30 pro cento minus que le pesos del testes in masculos de controlo de comparabile etates. Esseva trovate nulle differentia in le concentration medie del sperma. Tamen, le irradiate animales produceva circa 22 pro cento minus semine e 35 pro cento minus sperma per ejaculo que le animales de controlo. Esseva observate nulle differentia in le concentration initial de fructosa in le semine o in le utilisation de oxygeno durante un periodo de incubation de 75 minutas.

Nutritional Muscular Dystrophy in Lambs—Importance of the Ewe's Diet at Certain Stages of Gestation and Lactation

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NUTRITIONAL muscular dystrophy ("white muscle" disease, "stiff lamb" disease) is a degenerative myopthy affecting calves and lambs usually in their first few weeks of life. The natural disease in the young animal is the direct result of certain types of feed provided to the dam. Different rations have been incriminated in various areas. Because the natural disease in the young animal occurs principally in those parts of the world where prolonged winter feeding is necessary, it was supposed that incidence and severity would vary directly with the total length of time that the dam is maintained on the dystrophy-producing ration.

Recent interest in possible causal factors in feeds responsible for the disease has prompted an inquiry into the possible significance of certain specific periods in pregnancy and lactation during which the nature of the dam's diet may be of critical importance. Experiments have been conducted in 2 lambing seasons (1959 and 1960) to clarify these aspects of the relationship between the ewe's diet and the occurrence of muscular dystrophy in her lamb.

MATERIALS AND METHODS

Types of Hay Used.—All hay used in this experiment was irrigated alfalfa hay harvested in 1958 and 1959. The hay was derived from 2 sources in the Gallatin Valley, Montana. One hay type, designated "G" hay, was obtained in each year from premises on which muscular dystrophy had never been known to occur. The other hay type, designated "K" hay, was derived in each year from ranch K on which a history of muscular dystrophy in livestock had been established. Hay from ranch K had been used in experiments with sheep at this laboratory in previous years and had resulted in a high but variable incidence of the disease.

Breeding and Management of the Experimental Flock.—The flock in which these studies were conducted was composed of ewes predominantly of Columbia and Rambouillet breeding and ranging from 3 to 7 years in age at the time of the experiments.

The composition, breeding procedure, and general management of the flock were essentially similar in the 2 years of the trial. The ewes were bred on pasture in November to Rambouillet and Targhee rams equipped with marking harnesses so that the breeding dates of the ewes could be determined within 3 to 5 days. As the ewes were bred, they were moved to corrals and allowed one of the types of hay described, at the rate of 5 lb. per head per day. As each ewe lambed, her ration of hay was increased to 7 lb. and for the first 3 postpartum weeks all ewes also received ¼ lb. of oats per day.

Experimental Lots.—This report describes 19 experimental lots of ewes employed in the 2-year period of the trial. Four lots, each containing 30 to 60 ewes, served as controls to which either the "G" or the "K" type of hay was fed throughout gestation and early lactation. In all other lots, each of which consisted of 20 to 25 ewes, the type of hay fed was changed at various stages of gestation.

In each year, the lots were made up as the ewes were bred so that each lot would include ewes with closely approximated breeding dates. In this way, an average breeding date for the ewes in a lot could be stipulated. In those lots in which the type of hay fed was changed at a particular stage of gestation, the date of the change was calculated from the lot's average breeding date. In these lots, changes in hay were made approximately on the 30th, 60th, 75th, 90th, or 120th days of gestation. In other lots, a change in the type of hay fed took place at lambing time. Table 1 lists all of the lots employed in both years and indicates the hay-feeding regimen to which each lot of ewes was subjected.

Diagnostic Methods.—Previous evidence has been presented regarding the excellent correlation that exists between elevations in the glutamic oxalacetic transaminase content of the serum and the presence of lesions of muscular dystrophy.[1-4] This test was therefore employed in this trial as an objective aid to diagnosis. Samples of serum were obtained from all lambs at 7, 14, and 21 days of age and the serum glutamic-oxalacetic transaminase (SGO-T) content determined by the method of Ca-

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baud *et al.*,[2] the result being expressed in micrograms of pyruvic acid formed per milliliter of serum in 20 minutes at 25 C. (SGO-T units). All lambs were examined at the time of blood sampling and records were made of the presence and severity of symptoms. Necropsies were performed on all lambs that died during the experiment.

Measurement of Incidence.—In all lots, lambs considered relevant to the experiment were those which survived for at least 21 days or died prior to that time and were shown at necropsy to be affected with muscular dystrophy. Lambs were classed as affected with muscular dystrophy if at any age their SGO-T value exceeded 200 units, and if this elevation was not subsequently accounted for by other lesions found at necropsy.

RESULTS

The incidence of muscular dystrophy in lambs of all lots both in 1959 and 1960 is presented (Table 1). In lambs of ewes fed "G" hay for the entire period of gestation and early lactation, the incidence of muscular dystrophy was zero in 1959 and 1.3% in 1960 (one lamb with an elevated SGO-T unit). Lambs of ewes fed "K" hay for this same period showed an incidence of muscular dystrophy of 46.0% in 1959 and 67.7% in 1960. From these results, it is evident that the occurrence of the disease in this experiment can be attributed to the feeding of "K" hay.

Lambs of ewes fed "K" hay throughout gestation but not after lambing showed an incidence of 11.8% in 1959 and 10.7% in 1960, yet no lambs were affected when the ewes were fed "K" hay through only the first 4 months of gestation. Lambs of ewes fed "K" hay in only the last 2 months of gestation in 1959 had an incidence of 4.7%, and lambs of ewes fed "K" hay in the last 2½ months of gestation in 1960 had an incidence of 11.5%. These results suggested that the "K" hay exerted an influence on the incidence of the disease when fed in late gestation.

The highest figures of incidence occurred when "K" hay was fed to ewes not only during gestation but also in early lactation. The importance of the early lactation feeding period was evident regardless of the nature of the gestation diet. Figure 1 compares the incidence of muscular dystrophy in lambs of ewes on 3 gestational hay-feeding regimens, each followed either by a "G" hay diet or by a "K" hay diet in early lactation. In 1959 and 1960, respectively, an incidence of 8.2% and 17.2% followed the feeding of "K" hay during only the 3 weeks of early lactation. When "K" hay was fed in early lactation as well as in the 2nd half of gestation in 1960, the incidence was 51.9%, yet the incidence was only 11.5% if the "K" hay in late gestation was followed by "G" hay in early lactation. Finally, the maximum incidence in each year (46.0 and 67.7%) was obtained when the feeding of "K" hay in early lactation followed an entire gestational feeding period on "K" hay. However, this in-

TABLE 1—Ewes' Gestational and Early Lactational Diets in 1959 and 1960 and Ensuing Muscular Dystrophy Incidence in Lambs

Year	Hay fed in various months of pregnancy					Hay fed in lactation	Relevant lambs born	Cases of muscular dystrophy	Muscular dystrophy incidence (%)
	1	2	3	4	5				
1959	G	G	G	G	G	G	37	0	0
1960	G	G	G	G	G	G	75	1	1.3
1959	K	K	K	K	K	G	34	4	11.8
1960	K	K	K	K	K	G	28	3	10.7
1959	K	G	G	G	G	G	25	0	0
1959	K	K	G	G	G	G	18	0	0
1960	K	K	K/G	G	G	G	19	0	0
1959	K	K	K	G	G	G	27	0	0
1959	K	K	K	K	G	G	30	0	0
1959	G	G	G	G	K	G	26	0	0
1959	G	G	G	K	K	G	29	2	4.7
1960	G	G	G/K	K	K	G	26	3	11.5
1959	G	G	K	K	K	G	27	0	0
1959	G	K	K	K	K	G	29	2	4.7
1959	G	G	G	G	G	K	24	2	8.3
1960	G	G	G	G	G	K	29	5	17.2
1960	G	G	G/K	K	K	K	27	14	51.9
1959	K	K	K	K	K	K	26	12	46.0
1960	K	K	K	K	K	K	62	42	67.7

"K" = hay from a ranch on which muscular dystrophy occurred. "G" = hay from ranches without a history of muscular dystrophy.

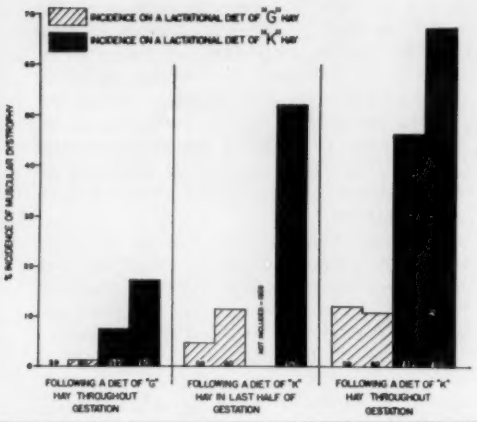
INCIDENCE ON A LACTATIONAL DIET OF "G" HAY
INCIDENCE ON A LACTATIONAL DIET OF "K" HAY
% INCIDENCE OF MUSCULAR DYSTROPHY
NOT INCLUDED IN 1959
FOLLOWING A DIET OF "G" HAY THROUGHOUT GESTATION
FOLLOWING A DIET OF "K" HAY IN LAST HALF OF GESTATION
FOLLOWING A DIET OF "K" HAY THROUGHOUT GESTATION

Fig. 1—Effect of lactational diet of the ewe on the incidence of muscular dystrophy in lambs. "K" hay was from a ranch on which muscular dystrophy occurred; "G" hay from ranches without a history of muscular dystrophy.

cidence was strikingly reduced in each year (11.8 and 10.7%) when the feeding of "K" hay terminated at lambing time.

DISCUSSION

The results of these experiments indicate that the incidence of muscular dystrophy in young lambs is not related simply to the total length of time for which the ewes are maintained on a dystrophy-producing ration. The feeding of such a ration particularly during late gestation and early lactation greatly increases the incidence of the disease in the lamb.

The effect of the early lactation diet alone is particularly striking. When ewes were fed the dystrophy-producing hay for only the short 3-week period of early lactation, the resulting incidence was at least as high as that occurring when the ewes were maintained on the hay for 5 months of gestation. Similarly, the incidence that resulted from the feeding of a dystrophy-producing ration throughout gestation was increased four- to sixfold when this ration was continued into the first 3 weeks of lactation.

A recent contribution from New Zealand[3] suggests tentatively that "white muscle disease" in lambs may be related to the management or nutrition of the ewes prior to breeding. This suggestion contrasts sharply with the conclusion reached in this experiment that the nutrition of the ewe in late gestation and early lactation exercises the greatest influence on the incidence of the disease. The condition referred to in New Zealand, however, was a congenital form of muscular dystrophy, affecting lambs at a much earlier age than in our experiment. It may be that the 2 conditions are etiologically distinct, even though their pathologic manifestations are similar.

Should the conclusions indicated by this experiment prove to have general application to the naturally occurring disease in sheep in this or other areas, certain practical possibilities come under consideration. The incidence of the disease in severely affected areas might be greatly alleviated by avoiding the use of locally produced hay crops in the feeding of late pregnant or lactating ewes, and substituting hay crops grown in unaffected localities. The feeding of the potentially dystrophy-producing hay only in the early months of gestation would apparently be a safe practice.

SUMMARY

Experiments have been carried out in which ewes were fed for various periods of gestation and early lactation on a dystrophy-producing hay and the ensuing incidence of muscular dystrophy in their lambs recorded.

Lambs remained unaffected when their dams received the hay in the 1st through the 4th months of gestation. Moderate incidence occurred when the hay was fed in the last 2 or 3 months of gestation.

The feeding of dystrophy-producing hay to ewes only in early lactation was followed by an incidence as high as that following feeding throughout gestation. The highest incidence occurred when dystrophy-producing hay was fed both in late gestation and early lactation.

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SUMMARIO IN INTERLINGUA

Dystrophia Muscular per Nutrition in Agnos. Importantia del Dieta del Oves Matre a Certe Stadios del Gestation e del Lactation

Esseva effectuate experimentos in que oves matre recipeva—durante varie periodos del gestation e del lactation initial—dietas de feno dystrophiogene, con registration del incidentia subsequente de dystrophia muscular.

Le agnos remaneva inafficite si le matres habeva recipite le feno durante le prime quatros menses del gestation. Un moderate incidentia de dystrophia muscular occurreva quando le feno esseva usate intra le ultime duo a tres menses del gestation.

Le administration de feno dystrophiogene al oves matre solmente durante le prime partes del periodo de lactation esseva sequite per un incidentia non inferior a illo resultante quando le administration esseva continuate durante le complete periodo de gestation. Le plus alte incidentia occurreva quando le feno dystrophiogene esseva usate al fin del gestation e etiam al initio del lactation.

Nutritional Muscular Dystrophy in Lambs—Administration of Selenium to Affected and Unaffected Lambs

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SELENIUM has been shown to reduce the incidence of muscular dystrophy in young animals when administered to the dam during pregnancy.[6, 8, 9, 13] It has also reduced the incidence of the disease when given to young lambs [3] and to young calves [11] before signs have appeared.

In New Zealand, the administration of selenium to older lambs affected with a condition known as "ill-thrift" reduced mortality and improved live-weight gains.[4, 5, 7] In one reported trial,[7] these effects were most prominent in a group of lambs that had been affected with "white muscle disease" prior to the administration of the selenium.

No account has been found describing the use of selenium in the treatment of overt clinical cases of muscular dystrophy. This report describes the results of administering selenium to young lambs affected with muscular dystrophy at the time of treatment, and to normal lambs of the same age.

MATERIALS AND METHODS

The lambs concerned in this report were born during an experiment in which ewes were fed, during various periods of their gestation and lactation, an irrigated alfalfa hay of known dystrophy-producing properties. This experiment has been described fully elsewhere.[14] Blood samples were taken from each surviving lamb at 7, 14, and 21 days of age. The serum glutamic oxalacetic transaminase (SGO-T) was determined by the method of Cabaud *et al.*,[2] and the results expressed in micrograms of pyruvic acid formed per milliliter of serum in 20 minutes at 25 C. (SGO-T units). The close correlation that exists between elevations in SGO-T and the presence of muscular dystrophy lesions in lambs has been reported.[1, 10]

The original purpose in administering selenium to lambs in this flock was to observe its effect upon live-weight gains. For this reason, in applying or denying selenium treatment to lambs, individuals were selected at random without regard to SGO-T levels, signs of muscular dystrophy, or the hay feeding regimen to which the dams had been subjected. Treated lambs were given a single dose of 1 mg. of selenium as sodium selenite in aqueous solution administered by subcutaneous injection on the 21st day of life. All lambs were weighed at that time.

After treatment had been applied in this random manner, it was decided to use the lambs also for observation on the effects of the treatment on the course and outcome of muscular dystrophy. For this purpose, all lambs were classified according to whether or not they were affected with muscular dystrophy. They were classed as affected if the SGO-T value at 7 or 14 days exceeded 200 units, and in most instances this objective criterion was supported by clinical evidence of the disease at the time of sampling. Lambs were classed as unaffected if at all times of sampling their SGO-T value had been less than 200 units.

Further progress of treated and untreated lambs was observed until the youngest lamb had attained an age of 6 weeks. All lambs dying in that period, and some that were killed after becoming totally disabled, were examined at necropsy.

RESULTS

The experiment included observations on 252 lambs, and the results are summarized (Table 1). Of 53 lambs that had evidence of muscular dystrophy by the age of 3 weeks, 15 had been given the selenium treatment. None of these lambs died within the period of experimental observation. The remaining 38 affected lambs had not been given selenium and, of these, 6 died naturally and 4 were killed *in extremis* at ages of 25 to 30 days. At necropsy, all of these 10 animals were found to be affected with gross generalized skeletal muscular dystrophy. One other lamb in the untreated group died at 43 days of age and was found to be affected with suppurative bronchopneumonia and myocarditis.

Of 199 lambs that had no evidence of muscular dystrophy by the age of 3 weeks, 78 had been given the selenium treatment. Eight of these lambs died naturally at ages of 28 to 46 days, and all were examined in

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TABLE 1—Effect on Mortality of Selenium Treatment of Clinically Dystrophic and Non-dystrophic Lambs

Treatment	Lambs with SGO-T* > 200 units			Lambs with SGO-T < 200 units		
	Total lambs	Deaths from M.D.**	Deaths from other causes	Total lambs	Deaths from M.D.	Deaths from other causes
Selenium†	15	0	0	78	0	8
No selenium	38	10	1	121	1	0

* SGO-T = serum glutamic oxalacetic transaminase. ** M.D. = muscular dystrophy. † Selenium treatment = a single subcutaneous injection of 1 mg. of selenium as sodium selenite in aqueous solution given at 21 days of age.

detail at necropsy. In 2 instances, death was evidently the result of peritonitis and septicemia, arising from a multifocal suppurative hepatitis in 1 case and from a perforating abomasal ulcer in the other. For the other 6 deaths in this group no satisfactory explanation could be found at necropsy. Three of these lambs had extensive retroperitoneal hemorrhage in the region of the abdominal aorta at the level of the renal arteries, and no evidence of traumatic injury could be found to account for these lesions. In the other 3 lambs, no specific lesions were observed.

Of the remaining 121 lambs that had no evidence of muscular dystrophy by 3 weeks of age and had not been given the selenium treatment, only 1 died within the period of experimental observation. This lamb died at 30 days of age and was found at necropsy to be affected with gross generalized skeletal muscular dystrophy. It is assumed that in this case the elevation in SGO-T commonly associated with these lesions had not yet taken place at the time the last blood sample was examined.

Discussion

While the prophylactic value of administering selenium to young animals exposed to dystrophy-producing conditions has already been reported,[3,11] it appears from the results reported here that selenium may also have therapeutic value in this disease. The mortality from muscular dystrophy in affected lambs not given selenium treatment exceeded 25%, while no affected lambs treated with selenium succumbed. Since the conditions of the trial did not permit prolonged clinical observation of affected animals, the rate of disappearance of the signs of the disability in treated and untreated subjects has not been recorded.

The significance of the deaths occurring in the group of lambs apparently unaffected with muscular dystrophy at the time of selenium treatment is uncertain. In these animals, the mortality was considerably greater than in untreated unaffected controls, and the equivocal nature of the necropsy findings in 6 of the 8 dead lambs promotes a consideration of the possible adverse or toxic effects of the single selenium treatment they were given.

At the time they reached 3 weeks of age, most lambs in this trial weighed between 15 and 25 lb. (7 to 12 kg.). The smallest lambs weighed 11 lb. (5 kg.). On a bodyweight basis, therefore, the dosage of selenium administered to the lambs was never more than 0.2 mg./kg. This figure is considerably lower than the minimum single parenteral doses of selenium reported as producing lethal effects in laboratory animals. These doses range, in milligrams of selenium as sodium selenite per kilogram of bodyweight, from 0.9 for the rabbit to 3.0 for the rat.[12] Similar information on the specific toxicity of sodium selenite administered by parenteral routes to lambs is not available. However, since the dosage used in this trial was relatively small, it seems unlikely that the increased mortality in unaffected selenium-treated lambs is attributable simply to selenium toxicity, and these deaths must at present remain unexplained.

Summary

In this experiment, 252 lambs were classed as affected or unaffected with muscular dystrophy on the basis of serum glutamic oxalacetic transaminase values determined at 7, 14, and 21 days of age. Some affected and some normal lambs at 3 weeks of age were given a single subcutaneous injection of sodium selenite in aqueous solution containing 1 mg. of selenium. In no case did this dosage rate exceed 0.2 mg. of selenium per kilogram of body weight. Mortality rates and causes of death were studied for at least 3 weeks after treatment was applied to the youngest lamb. No deaths occurred in 15 affected lambs given

the selenium treatment, although 38 untreated affected lambs showed a 25% mortality from the disease.

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SUMMARIO IN INTERLINGUA

Dystrophia Muscular Nutritional in Agnos. Le Administration de Selenium a Agnos Afficite e a Agnos Non-Afficite

In iste experimento, 252 agnos esseva classate como afficite o non-afficite de dystrophia muscular super le base de determinationes del valores pro transaminase glutamico-oxaloacetic del sero al etates de 7, 14, e 21 dies de etate. Plures del afficite e plures del non-afficite agnos recipeva, al etate de tres septimanas, un sol injection subcutanee de selenito de natrium in un solution aquose que contineva 1 mg de selenium. Iste injection non amontava in ulle del casos a plus que un dosage de 0,2 mg de selenium per kg de peso corporee. Le mortalitate e le causas de morte esseva studiate durante al minus tres septimanas post le application del tractamento al plus juvene del agnos. Nulle mortes occurreva inter 15 afficite agnos que recipeva le tractamento a selenium, durante que un gruppo de 38 afficite sed non-tractate agnos habeva un mortalitate de 25 pro cento ab dystrophia muscular.

Nutritional Muscular Dystrophy in Lambs—Effect of Administering Selenium to Pregnant Ewes

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CERTAIN differences exist in the clinical features and predisposing dietetic conditions that characterize the naturally occurring degenerative myopathies of young animals described in various parts of the world under the name "white muscle disease." For example, the feeds held responsible for the disease often have little in common, the age at which clinical signs first appear differs in some areas from others, and the response of the condition to vitamin E therapy varies widely from place to place. These differences make it necessary to compare the results obtained in various areas.

The value of the administration of selenium to ewes as a preventive for nutritional muscular dystrophy in lambs has already been established in certain areas.[3-5] This report describes a study of muscular dystrophy in lambs under Montana conditions in which selenium was administered by injection to ewes maintained on a particular hay diet.

MATERIALS AND METHODS

Management and Feeding of Sheep.—Columbia and Rambouillet ewes, 3 to 7 years old, were exposed for pasture breeding in early November, 1959, to Targhee and Rambouillet rams. The rams were equipped with ewe-marking harnesses so that bred ewes could be identified immediately and removed to corrals for hay feeding. The hay used was from the 1st and 2nd cuttings of an irrigated alfalfa crop grown in 1959 on a ranch where muscular dystrophy had occurred in lambs in previous years. From breeding until lambing time, all ewes were given hay alone, fed at all times as an equal mixture of 1st and 2nd cuttings. From lambing time and for 3 weeks postpartum, the hay ration was supplemented with oats fed at the rate of 0.25 lb. per head per day. Shortly after the lambs reached 3 weeks of age, they were turned out to pasture with the ewes.

Experimental Lots.—The 84 ewes that lambed during this experiment were distributed in 2 lots. Selenium was administered to 33 ewes of 1 lot in 4 subcutaneous injections, each containing 10 mg. of selenium as sodium selenite in aqueous solution. These injections were given in early January, February, and March, 1960, and finally to each ewe at the time she lambed in April. The 51 ewes in the other lot were given no selenium and served as controls.

Diagnostic Methods.—Previous evidence,[1,6] has shown that an excellent correlation exists between the presence of muscular dystrophy lesions and elevations in the serum glutamic oxalacetic transaminase (SGO-T). Consequently, the test for SGO-T has been employed as an objective aid to diagnosis in this trial. Samples of serum were obtained from all surviving lambs at 7, 14, and 21 days of age and the SGO-T content determined by the method of Cabaud *et al.*,[2] the result being expressed in micrograms of pyruvic acid formed per milliliter of serum in 20 minutes at 25 C. (SGO-T units). Elevations of the SGO-T value above 200 units were regarded as indicative of the presence of muscular disease. In addition, all lambs were examined clinically at the time of blood sampling and records made of the presence and severity of clinical signs. Necropsies were performed on all lambs that died or were killed in the experiment.

RESULTS

The results of the trial are summarized (Table 1). Of the 78 lambs born to ewes not given selenium, 42 had SGO-T values exceeding 200 units within the first 3 weeks of life. More than half of these lambs had this elevation by the 14th day of life, and prominent signs of stiffness were seen in almost all cases. Of these experimental lambs, 18 either died or were killed. Ten died naturally, with muscular dystrophy; and another 7 were killed, generally *in extremis* and, at necropsy, were found to have the disease. One lamb died as a result of suppurative myocarditis.

Of the 51 lambs born to ewes given selenium, only 6 had SGO-T values exceeding 200 units. In only one instance had this elevation become evident by the 14th day of life; in this particular lamb, the SGO-T level had

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TABLE 1—Effect, on Muscular Dystrophy in Lambs, of Selenium Given to Ewes

Effect	Ewes not given selenium	Ewes given selenium*
Number of lambs born	78	51
Lambs with SGO-T** > 200 units at any age	42	6
Lambs with SGO-T > 200 units at 7 days of age	7	0
Lambs with SGO-T > 200 units at 14 days of age	28	1
Lambs with SGO-T > 200 units at 21 days of age	29	5
Natural deaths, with lesions of muscular dystrophy at necropsy	10	0
Lambs killed after elevation of SGO-T	7	4
Lambs with lesions of muscular dystrophy; killed after elevation of SGO-T	7	1

* Selenium administered subcutaneously to ewes as sodium selenite in aqueous solution (10 mg. Se) in each of the last 3 months of pregnancy and at lambing time.
** Serum glutamic oxalacetic transaminase.

returned to normal by the 21-day sampling. This lamb remained clinically unaffected throughout the experiment. None of the 6 lambs had signs of the disease, and none died naturally. Four of the 6 were killed and examined at necropsy, but gross lesions of muscular dystrophy were observed in only 1; the other 3 lambs appeared grossly normal. Unfortunately, muscle tissues from these 3 lambs were not retained for histologic examination.

No signs of toxicosis followed the administration of selenium to the ewes.

DISCUSSION

The administration of selenium to ewes during pregnancy greatly reduced, though did not entirely eliminate, the incidence of detectable muscular dystrophy in lambs. In this respect, the experiment confirmed that results previously obtained by others [3-5] are apparently applicable to "white muscle disease" as it occurs in Montana.

The data pertaining to the 6 lambs in the selenium-treated group that had significant elevations in SGO-T are particularly interesting. In only 1 of these was the presence of muscular dystrophy later confirmed by the demonstration of lesions of the disease at necropsy. None of the lambs developed clinical signs, and 3 were found grossly normal at necropsy. It may be argued that 5 of them were not, in fact, affected with the disease, but no alternative explanation for the elevated SGO-T levels was found. Previous application of this test to over 1,000 young lambs [6,7] has shown it to be a remarkably sensitive and early indicator of the presence of muscular dystrophy. Only 2 other conditions of the young lamb, *i.e.*, suppurative polyarthritis and suppurative myocarditis, are believed to have been responsible for elevations in SGO-T in the 3 years that the test has been in use at this laboratory. In the present trial, no other instances arose in which an apparent conflict occurred between a lamb's SGO-T value and the presence of signs or lesions.

It may be that the elevations in SGO-T observed in these 6 lambs reflected the onset of a dystrophic process in their muscles. Clinical signs and gross necropsy lesions may have been less evident than in affected lambs of ewes not given selenium because the disease was delayed in onset and was more mild.

SUMMARY

Subcutaneous injections of aqueous sodium selenite, each containing 10 mg. of selenium, were given in each of the last 3 months of gestation and at lambing time to ewes maintained on a dystrophy-producing ration. No signs of toxicosis were observed. The incidence of muscular dystrophy in the lambs of these ewes was greatly reduced when compared with that in control lambs, though not entirely eliminated. Evidence suggested that, in those affected lambs of selenium-treated ewes, the onset of the disease may have been delayed and its character made less severe.

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SUMMARIO IN INTERLINGUA

Dystrophia Muscular Nutritional in Agnos. Effecto del Administration de Selenium a Oves Pregnante

Injectiones subcutanee de aquose selenito de natrium, continente 10 mg de selenium cata un, esseva administrate in cata un del tres ultime menses del gestation e al tempore del agnellamento a oves matre que habeva essite mantenite con dietas de virtute dystrophiogene. Nulle signos de toxicosis esseva notate. Le incidentia de dystrophia muscular in le agnos de iste oves matre esseva grandemente reducite in comparation con agnos de controlo, sed illo non esseva eliminate completemente. Certe observationes suggereva que le agnos afficite ben que parturite ab matres tractate con selenium habeva un retardo in le declaration del morbo e que in illos le character del morbo esseva minus sever.

Serum and Tissue Residues Following Selenium Injections in Sheep

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Reno, Nevada

RECENT REPORTS [2,4,5] indicate the usefulness of selenium (Se) in preventing white muscle disease in sheep. McLean *et al.*[3] found that, in addition to preventing white muscle disease, selenium is necessary for normal metabolism and may improve weight gains and wool production. With the increasing evidence of selenium's value in sheep, questions occur concerning its mode of administration, absorption time, metabolism, and possible tissue residues. In view of its toxic nature, the use of selenium in animals to be used for human consumption may be a public health hazard in the event selenium is stored or accumulated in tissues of treated animals.

This study was undertaken to determine selenium serum levels over a 5-month period in ewes given injections of sodium selenite (Na_2SeO_3) and barium selenate ($BaSeO_4$). At the conclusion of this time, all animals were slaughtered to determine residues of injected selenium in tissue. In view of the apparent value of selenium in preventing white muscle disease it was also desirable to determine if a low level of selenium could be maintained over an extended period of time as a result of a single injection. A further objective was to determine toxic and safe levels of sodium selenite and barium selenate when injected subcutaneously, and to measure selenium concentrations in serum and tissues following injections of lethal and sublethal amounts.

MATERIALS AND METHODS

Twelve ewes were allotted to 3 groups of 4 each and were given subcutaneous injections of 2 levels of radioactive barium selenate and 1 level of radioactive sodium selenite.

Sodium selenite was prepared with selenium^{75} from selenous acid by neutralizing it with sodium hydroxide and adding sodium selenite carrier. Barium selenate was prepared by oxidizing selenous acid (containing Se^{75}) with 30% hydrogen peroxide, adding sodium selenate carrier, and precipitating barium selenate by the addition of barium chloride after adjusting to approximately pH 10. The radioactive barium selenate was filtered and washed with small amounts of water and with acetone. Both sodium selenite and barium selenate were suspended in hot peanut oil containing 2% beeswax. The barium selenate was prepared so that each milliliter of the oil-beeswax mixture contained 200 mg. of the salt. The sodium selenite was prepared so that each milliliter of oil-beeswax mixture contained 5 mg. of the salt.

Group I consisted of 1 Hampshire and 3 Rambouillets, averaging 164 lb. of weight and 5 years of age. Each was given 5 mg. of sodium selenite per 100 lb. body weight subcutaneously. In 2 animals it was injected in the ear while 2 were given the injections in the shoulder.

Group II consisted of 1 Hampshire, 2 Rambouillets, and 1 Columbia, averaging 175 lb. of weight and 7 years of age. Each was given 50 mg. of barium selenate per 100 lb. of body weight subcutaneously. Two animals were given subcutaneous injections in the ear and 2 were given the injection in the shoulder.

Group III consisted of 1 Hampshire, 2 Rambouillets, and 1 Columbia, averaging 160 lb. of weight and 6 years of age. Eeach was given 20 mg. of barium selenate per 100 lb. of body weight subcutaneously. Two animals were given injections in the ear and 2 were given injections in the shoulder.

Serum samples to determine levels of injected selenium were collected from all animals the day after injections and thereafter at weekly intervals to 140 days. The radioactive selenium content of the samples was measured by counting the 0.269-m.e.v. *gamma* ray. A measured serum sample, usually 5 ml., was placed in a plastic tube and counted with a well-type scintillation detector, single channel pulse-height analyzer, and scaler. The samples were counted for 1,000 counts or 15 minutes, whichever came first, or, in the case of very radioactive samples, for at least 1 minute. After determining the counts per minute less background, measurements were compared to a selenium^{75} standard calibrated in terms of the injected material to determine serum levels of selenium resulting from injections of radioactive material. The values of serum selenium are expressed as micrograms of selenium per 100 ml. serum.

The experiment was terminated 148 days after selenium injection and all animals were slaughtered. At that time, tissue samples were collected, weighed

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(usually 5 to 7 Gm.), placed in plastic counting tubes, frozen, and, at a later date, counted in the same manner as described for serum to determine residual radioactive selenium. Counts per minute less background were determined and then compared to the selenium[75] standard to determine micrograms of selenium per 100 Gm. of tissue.

Eight sheep, 2 Rambouillets, 5 Hampshires, and 1 Columbia were injected with 4 levels of sodium selenite to measure toxicity of this material (Table 2).

Radioactive sodium selenite was suspended in peanut oil containing 2% beeswax as previously described and injected subcutaneously in the shoulder. Injections were made at levels of 20, 40, 80, and 150 mg./100 lb. of body weight. Serum measurements of radioactive selenium were made at the time intervals indicated (Table 2).

Selenium residues were determined for tissues indicated (Table 3) in 1 of the 2 animals given 20 mg., 40 mg., and 80 mg. of sodium selenite per 100 lb. of body weight, 7 days after injections. Selenium residues were also determined in tissues of sheep 58 C, which died 18 to 20 hours after injection of sodium selenite at the 80-mg. level.

Radioactive barium selenate was prepared for similar toxicity studies. Levels of 50, 100, 200, and 400 mg./100 lb. of body weight were injected subcutaneously into each of 4 sheep. A 5th animal was given an injection of nonradioactive barium selenate at the rate of 1,080 mg./100 lb. of body weight.

RESULTS

Serum selenium levels following the injection of 5 mg. of sodium selenite per 100 lb. of body weight in ewes of group I are recorded for the 140-day observation period (Fig. 1). Serum selenium levels of animals given injections in the ear and those given injections in the shoulder were not significantly different. For this reason, selenium levels of all 4 animals were included in the averages which are recorded. The greatest serum concentration of injected selenium occurred 1 day following injection, and this decreased rapidly up to 14 days when there appeared to be a leveling-out of serum concentrations at a relatively low level. On day 1, the level was 10 μg. of selenium per 100 ml. of serum. On day 7, the level was down to 5 μg. and, by day 14, the measured amount was 4 μg./100 ml. of serum. A correlation between serum selenium levels and days following injection occurred. A significant regression coefficient of -0.03881 was determined. The regression line plotted (Fig. 1) indicates a disappearance of serum selenium 123 days after injection. Even though measurements of 0.72, 0.61, and 0.56 μg./100 ml. of serum were made after 123 days, it is doubtful if such small quantities can be considered significant.

Serum selenium levels of ewes given 50 mg. of barium selenate per 100 lb. of body weight (group II) are recorded (Fig. 2). Animals in group II, given injections in the ear, developed significantly lower serum selenium levels during the period of observation when compared to levels in animals given injections in the shoulder. For this reason they are recorded separately. The highest selenium level following injection in the shoulder occurred between 7 and 21 days, with levels reaching 16, 15, and 16 μg./100 ml. of serum on days 7, 14, and 21, respectively. On day 1, the level was only 4 μg./100 ml. of serum. On day 140, the level was 4 μg./100 ml. of serum. Animals given injections in the ear had slightly

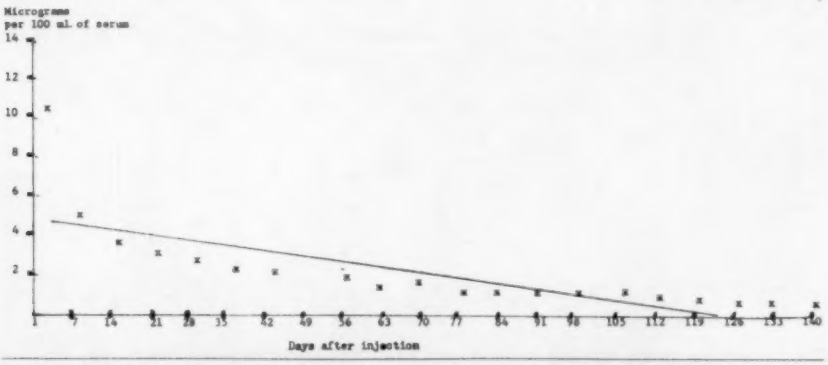

Fig. 1—Average serum selenium levels in group I ewes following injections with sodium selenite at the rate of 5 mg./100 lb. of body weight. The line indicates the linear regression of selenium levels (regression coefficient = −0.03881, highly significant); x indicates the average selenium level.

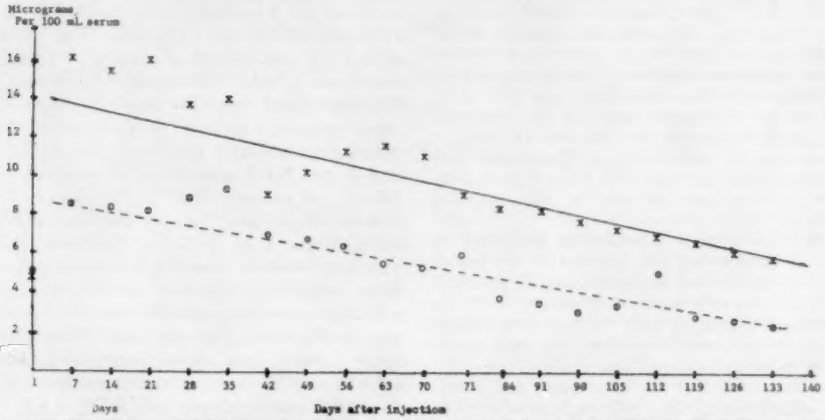

Fig. 2—Average serum selenium levels in group II ewes following injections with barium selenate at the rate of 50 mg./100 lb. of body weight. Injections in the shoulder: line indicates linear regression of serum selenium levels (regression coefficient = −0.06138) and x indicates average selenium level. Injections in the ear: broken line indicates linear regression of serum selenium levels (regression coefficient = −0.04658 highly significant) and O indicates average selenium level.

slower absorption, in that the highest serum selenium level was not reached until day 35, when 9 μg./100 ml. of serum was measured. After 140 days, the selenium levels of these animals had decreased to 2 μg./100 ml. of serum. A significant correlation occurred between the serum selenium levels and the days after injection. The regression coefficient was −0.0614 for those given injections in the shoulder and −0.0466 in animals given injections in the ear. The regression lines are recorded (Fig. 2).

Measurements of serum selenium levels occurring in ewes of group III after 20 mg. of barium selenate was administered are recorded (Fig. 3). Animals in group III that were given injections in the ear developed significantly lower serum selenium levels during the period of observation when compared to levels in animals given

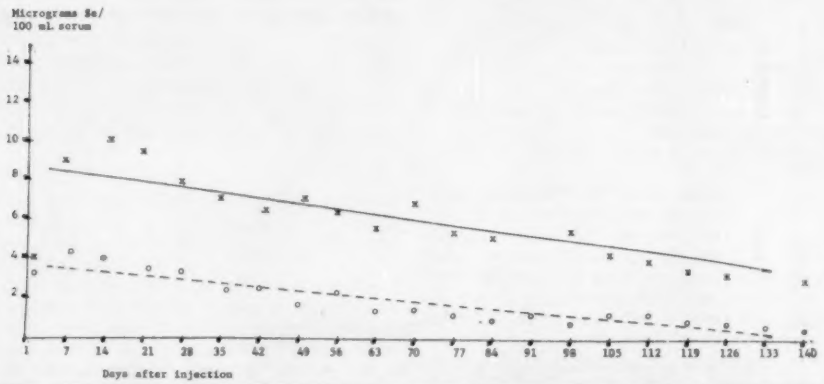

Fig. 3—Average serum selenium levels in group III ewes following injections with 20 mg./100 lb. of body weight. Injections in the shoulder: line indicates linear regression of serum selenium levels (regression coefficient = −0.03760 highly significant) and x indicates average selenium level. Injections in the ear: broken line indicates linear regression of serum selenium levels (regression coefficient= −0.02463 highly significant) and O indicates average selenium level.

injections in the shoulder. For this reason, they are recorded separately. The highest selenium levels after injection in the shoulder occurred between 7 and 21 days. Levels of 10 μg./100 ml. of serum occurred at days 7, 14, and 21, respectively. At the conclusion of the test period 140 days after injection, a selenium level of 3.0 μg./100 ml. of serum was measured. Serum levels following injections in the ear were highest 7 days after injection, reaching 4 μg./100 ml. of serum. At the conclusion of the experiment 140 days after the injection, serum levels had decreased to 0.6 μg./100 ml. A significant correlation occurred between the serum selenium level and the days after injection. The regression coefficient in animals given injections in the shoulder was −0.0376; it was −0.0246 after injection in the ear. The regression lines are recorded (Fig. 3).

In addition to differences in serum selenium levels of animals given barium selenate via the ear and shoulder, other significant differences occurred. In animals given barium selenate in the shoulder, injections of 50 mg./100 lb. of body weight resulted in significantly higher serum selenium levels than injections of 20 mg./100 lb. of body weight. Serum selenium levels in animals given 20 mg. of barium selenate in the shoulder were significantly higher than serum levels in animals given 50 mg. of barium selenate in the ear. Even though serum selenium levels in animals given 50 mg. in the ear were significantly greater than levels in animals given sodium selenite, differences between animals given 20 mg. of barium selenate in the ear and animals given sodium selenite were not significant.

Tissue residues of injected selenium, 148 days after injection, are expressed as micrograms per 100 Gm. of tissue (Table 1). Generally, tissue selenium levels paralleled the serum levels (Fig. 1–3). Animals with low serum levels had correspondingly low tissue residues. Under the conditions of our experiments, selenium appeared to be present in widely varying amounts, depending on the tissue being examined. Using an average of tissue levels from all animals injected with selenium, it is possible to arbitrarily divide tissues into 3 groups, those having low, medium, and high selenium residue. Those tissues in the low-residue group (up to 2.5 μg./100 Gm.) were bone, skin, skeletal muscle, fat, serum, and bile. Tissues in the medium-residue group (2.6–5.79 μg./100 Gm.) were hoof, wool, liver, small intestines, and whole blood. Tissues having high selenium residue (5.80 μg. and higher per 100 Gm.) were spleen, heart, lung, brain, and kidney. The tissue consistently having the highest concentration of selenium 148 days after injection was kidney, and the lowest was fat and bone.

The results of toxicity studies of sodium selenite are recorded (Tables 2, 3). Both animals given sodium selenite at the rate of 150 mg./100 lb. of body weight died within 20 hours after injection. One of the 2 animals given sodium selenite at the rate of 80 mg./100 lb. of body weight died approximately 18 hours after injection. The 2 animals given 40 mg./100 lb. and the 1 remaining animal given 80 mg./100 lb. of body

TABLE 1—Average Tissue Residues of Selenium (μg./100 Gm. of Tissue) 148 Days After Injection with Sodium Selenite and Barium Selenate

	Group I (5 mg. sodium selenite)		Group II (50 mg. barium selenate)		Group III (20 mg. barium selenate)		
	Ear	Shoulder	Ear	Shoulder	Ear	Shoulder	
No. of animals	2	2	2	2	2	2	Average of all treatments
Bone	0.18	0.18	0.45	1.03	0.22	0.54	0.43
Hoof	2.01	2.68	4.16	5.83	2.44	6.85	4.00
Skin	0.64	0.64	2.73	3.83	0.66	3.72	2.04
Wool	2.65	2.08	16.69	6.04	2.61	3.86	5.66
Heart	1.69	2.26	5.61	12.82	1.87	10.53	5.80
Muscle	0.46	0.56	1.48	3.50	0.46	2.48	1.49
Spleen	2.38	2.53	9.40	13.68	2.24	12.85	7.18
Liver	1.52	1.21	5.15	9.67	1.31	6.51	4.23
Lung	1.66	2.24	6.81	12.58	1.67	10.98	5.99
Kidney	12.26	17.19	42.34	57.50	11.55	52.29	32.19
Small intestine	2.21	2.08	7.70	8.64	2.07	9.50	5.37
Bile	0.06	0.04	0.77	1.37	0.05	0.29	0.43
Whole blood	1.08	2.31	4.42	12.93	1.47	8.13	5.06
Serum	0.50	0.62	1.76	4.25	0.60	3.00	1.79
Fat	0.18	0.12	0.49	0.74	0.11	0.58	0.37
Brain	2.26	2.19	5.70	7.50	2.28	15.86	5.96

TABLE 2—Serum Selenium Levels (μg./100 ml. of Serum) Following Lethal and Sublethal Injections of Sodium Selenite

Animal No.	Dosage (mg./100 lb. of body weight)	Time interval after injection						
		2 hr.	6 hr.	22 hr.	30 hr.	2 days	3 days	6 days
11 H*	20	45	50	13	14	10	9	10
107 R	20	59	55	30	23	14	11	12
151 H*	40	100	93	31	23	15	15	15
140 R	40	96	87	33	29	16	12	14
107 H*	80	203	239	132	97	50	35	28
58 C**	80	110	100	D†	....	....	....	....
41 H	150	412	408	D‡	....	....	....	....
25 H	150	355	177	D§	....	....	....	....

* Killed 7 days after inoculation for tissue residue studies. ** Tissues were collected 3 to 4 hours after death for residue studies. † Death occurred approximately 18 hours after injection. ‡ Death occurred approximately 20 hours after injection. § Death occurred approximately 7 hours after injection.

weight went off feed for 24 hours but recovered without evidence of other toxic effects. Neither animal given 20 mg./100 lb. of body weight had signs of toxic reaction after injections.

Serum selenium levels (Table 2) were generally higher in those animals given the larger injections. The exception was animal 58 C which died 18 to 20 hours after injection with the 80-mg. level of sodium selenite. Serum levels in ewe 58 C were much lower than in ewe 107 H, given the same amount of selenium, and practically the same as in animals given only the 40 mg. level of sodium selenite.

Tissue selenium residues of 3 animals, given 20, 40, and 80 mg. of sodium selenite per 100 lb. of body weight, were measured 7 days after injection and recorded (Table 3). In addition, the tissue residues are recorded on animal 58 C, that died 18 to 20 hours after selenium injection. Tissue levels of selenium in most instances appeared to correspond to the amounts of material injected. The exception was animal 58 C. The most striking differences between tissue levels of ewe 58 C (20 hours after injection) and the other 3 animals (7 days after injection) occurred in selenium levels in the liver, kidneys, and heart. Animal 58 C had a lower selenium level in the liver, and much higher levels of selenium in the heart and kidneys than the other 3 animals.

At necropsy of the 3 animals given lethal doses of sodium selenite and the 3 animals killed 7 days after injection, there was no evidence of gross pathologic changes that could be attributed primarily to the selenium injections, other than evidence of tissue irritation with edema and swelling at the site of injection.

The 5 sheep given barium selenate at the rate of 50, 100, 200, 400, and 1,080 mg./100 lb. of body weight had no signs of toxic reactions other than local swelling at the site of injection. The maximum serum selenium levels, measured in animals given 50, 100, 200, and 400 mg. of barium selenate per 100 lb. of body weight were

TABLE 3—Tissue Selenium Levels (μg./100 Gm. of Tissue) Following Lethal and Sublethal Injections of Sodium Selenite

Tissue	11 H—Injected with 20 mg./100 lb. body weight*	151 H—Injected with 40 mg./100 lb. body weight*	107 H—Injected with 80 mg./100 lb. body weight*	58 C—Injected with 80 mg./100 lb. body weight**
Bone	2	2	6	14
Hoof	7	11	48	21
Skin	8	7	22	42
Wool	53	9	45	4
Heart	7	12	31	89
Muscle	2	3	6	15
Spleen	16	12	85	51
Liver	223	420	774	252
Lung	14	17	47	41
Kidney	72	122	129	431
Small intestines	12	17	25	39
Bile	4	11	8	22
Fat	1	1	2	10
Brain	3	4	12	14

* Tissue samples were taken 7 days after selenium injections.
** Tissue samples on 58 C were taken approximately 4 hours after death, which occurred about 20 hours after selenium injection.

25, 33, 30, and 11 μg./100 ml. of serum, respectively.

DISCUSSION

The relatively high absorption rate of sodium selenite is clearly demonstrated by the rapid increase in serum selenium only 1 day after injection. This is followed by rapid reduction in the selenium level. The minimum serum selenium level for preventing white muscle disease is not known, but less than 1 or 2 μg./100 ml. or 0.01 p.p.m. would probably be ineffective. The disappearance of selenium when injected as a sodium salt in the amounts recorded in this experiment is relatively rapid and probably would result in serum levels too low to prevent white muscle disease after about 40 days. The sodium salt would, however, appear to be satisfactory for lambs. If protection from white muscle disease is related to serum selenium levels, then protection would occur immediately and last 30 to 40 days, carrying the animal through the most dangerous period. The rapid elimination reduces the possibility of public health hazards from high tissue residues.

The insoluble barium selenate appears to be absorbed more slowly, maintaining serum selenium levels over a longer time. The barium salt is also relatively nontoxic, making it a safe material to use with respect to toxicity in animals being treated. These characteristics partially explain the effectiveness of barium selenate in preventing white muscle disease when injected into the ewes prior to parturition. A dose of 40 mg. of barium selenate per 100 lb. of body weight in the ewe has been shown to be effective in preventing white muscle disease,[1] and it is possible that 20 mg./100 lb. of body weight may also be effective in view of the low regression coefficient and the sustained serum levels (Fig. 3). Even though lower serum levels accompanied the lower level of dosage, the reduction in selenium recovered was not proportional to the amount of the original injection.

In tissue studies made 148 days after injection of selenium salts at levels previously demonstrated to prevent white muscle disease, residual selenium was extremely low. Underwood[6] reports selenium levels in wheat (dry basis) ranging from 4 to 40 p.p.m. and levels in meat ranging from 1 to 8 p.p.m. in seleniferous areas. An average selenium tolerance of 3 to 4 p.p.m. for man is suggested by Underwood.[6] Tissue selenium resdiues measured in sheep injected with sodium and barium selenium salts were far below 3 to 4 p.p.m. Kidneys contain several times more residual selenium than other organs. At the highest level measured during these studies (other than toxicity trials), kidneys were found to contain 57.5 μg./100 Gm. of tissue. This, converted to p.p.m., would be 0.575 on the basis of wet weight. Hawk *et al.*[1] report that normal kidneys contain 79.47% water, so, on the basis of dry weight, it would be possible to recover 2.87 p.p.m. of selenium. A similar conversion for muscle on a dry weight basis would yield 0.174 p.p.m. of selenium residue 148 days after the injection of 50 mg. of barium selenate per 100 lb. of body weight. Selenium residues of other organs are easily calculated as parts per million and would be even lower than this in instances where either sodium selenite or 20 mg. of barium selenate were injected.

Selenium residues in skeletal muscle, 7 days after the injection of near-toxic levels of sodium selenite, were only 0.1 to 0.3 p.p.m.

Toxicity studies revealed considerable individual variation in toxic levels of selenium. Dosage at 150 mg. of sodium selenite per 100 lb. of body weight was clearly toxic; however, only 1 of 2 animals died after being given the 80-mg. level. The animal that died had a serum selenium level equal to that of animals given the 40-mg. level. An assumption may be made on this basis that some animals are more susceptible to the toxic effects of selenium than others. On the basis of the equal serum selenium levels and the observation of a transient anorexia, it is possible that a sodium selenite level of 40 mg./100 lb. of body weight might prove lethal to some animals. Further studies to determine the significance of the high selenium level in the heart and kidneys accompanied by a lower level in the liver of the 1 animal that died are indicated.

SUMMARY

Observations have been made on serum selenium levels and tissue residues following the subcutaneous injection of radioactive sodium selenite at the rate of 5 mg. per 100 lb. of body weight and radioactive barium selenate at the rate of 20 and 50 mg. per 100 lb. of body weight. Injections were made in the shoulder and in the ear.

The sodium salt was found to be absorbed and eliminated more rapidly than the ba-

rium selenate. Measurable selenium tissue residues, recorded 148 days after injections, corresponded with serum levels. The highest selenium residues were found in kidneys, with relatively decreasing amounts seen in spleen, lungs, brain, heart, and wool. Fat and bone had the lowest accumulations of selenium. With barium selenate, the site of injection influenced serum and tissue levels. Injections in the ear were absorbed less efficiently than injections in the shoulder.

Toxicity experiments were conducted with barium selenate and sodium selenite. Sodium selenite was found to be lethal at 80 mg. per 100 lb. of body weight, and it was found to produce toxic reactions at 40 mg. per 100 lb. of body weight. Barium selenate, at the rate of 1,080 mg. per 100 lb. of body weight, was nontoxic other than causing a local reaction at the site of injection.

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SUMMARIO IN INTERLINGUA

Residuos Seral e Tissular post Injectiones de Selenium in Oves

Esseva investigate le nivellos seral de selenium e le residuos tissular de selenium post injectiones subcutanee in oves de radioactive selenito de natrium in un dosage de 5 mg per 100 lbs de peso corporee e de radioactive selenato de barium in dosages de 20 e 50 mg per 100 lbs de peso corporee. Le injectiones esseva effectuate in le spalda e in le aures.

Esseva trovate que le sal de natrium esseva absorbite e eliminate plus rapidemente que le sal de barium. Le mensurabile residuos tissular de selenium, registrate 148 dies post le injection, esseva in correspondentia con le nivellos seral. Le plus alte concentrationes de residuo de selenium esseva trovate in le renes, con quantitates relativemente decrescente etiam incontrate in splen, pulmon, cerebro, corde, e lana. Grassia e osso habeva le plus basse accumulationes de selenium. In le caso de selenato de barium, le sito del injection exerceva un influentia super le nivellos seral e tissular. Injectiones in le aures esseva absorbite minus efficacemente que injectiones in le spalda.

Experimentos de toxicitate esseva effectuate pro selenato de barium e pro selenito de natrium. Essava constatate que selenito de natrium es letal in un dosage de 80 mg per 100 lbs de peso corporee. Illo produce reactiones toxic al nivello de 40 mg per 100 lbs de peso corporee. Selenato de barium, in un dosage de 1.080 mg per 100 lbs de peso corporee, esseva nontoxic, a parte le causation de un reaction al sito del injection.

Enterotoxemia of Sheep. I. Effects of *Clostridium perfringens* Type D Toxin on the Brains of Sheep and Mice

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Clostridium perfringens type D is generally recognized to be the etiologic agent in enterotoxemia of sheep; however, little is known regarding the action of its toxins on the tissues of affected animals. The principal sign of the disease is the convulsive seizure which strongly suggests that the toxin acts on the tissues of the central nervous system. The absence of knowledge regarding the pathologic effects of type D toxin on the brain of affected animals prompted the author to conduct this study.

Review of Literature

Clostridium perfringens type D, *epsilon* toxin, is considered to be the cause of enterotoxemia in sheep; it is believed that affected animals die as the result of absorption of toxin produced by the organism in the alimentary tract,[1, 3, 7, 13] and that overeating is a predisposing factor in the production of toxin.[4]

Gill[7] recognized 3 clinical types of enterotoxemia: (1) a hyperacute convulsive type in which the lambs sickened suddenly, became convulsed, and died within a few hours; (2) an acute head-retracted form similar to the preceding type but in which the clinical signs were less acute; and (3) a subacute lethargic type characterized by depression, dullness, and impaired vision. Gill was unable to conclusively prove that each of the 3 types was caused by type D toxins. In his final view, only the hyperacute convulsive form represented the true enterotoxemia. In sections of brains from lambs with hyperacute enterotoxemia, he found congestion and edema of the meninges.

Kellaway *et al.*[12] studied the neurotoxic effects of *Cl. perfringens* type D toxin, and observed that, in unanesthetized lambs given intravenous doses of toxin slightly in excess of the minimum lethal dose (MLD), the blood sugar was markedly increased. Also noticed was dyspnea suggestive of possible over-ventilation, resulting in a decreased alkali reserve. They concluded that the clinical signs resulted from action of the toxin on the central nervous system. The brains and spinal cords of 2 of their lambs were examined macroscopically and microscopically, but lesions were not discernible.

During the period 1950 to 1955, Hartley[9] performed necropsies on 36 lambs which had a wide variety of neurologic signs. Many of the lambs came from premises where serious epizootics of enterotoxemia were reported or suspected. Macroscopic examination of the brains revealed bilateral focal symmetrical encephalomalacia (FSE) in the region of the internal capsule adjacent to the basal ganglia, lateral to the iter, beneath the posterior colliculi, and in the cerebellar peduncles. Hartley allotted the lambs to 4 groups on the basis of duration of the neurologic signs. Four lambs in group I were found dead, and *Cl. perfringens* type D toxin, was demonstrated in the ileal contents of only 1 lamb. Group II contained 24 lambs which had been affected for 1 to 6 days. Ileal contents of 9 of these lambs were subjected to examination for *Cl. perfringens* type D toxin, with positive results in 3. Hartley believed that the demonstration of type D toxin in the ileal contents of these lambs is not sufficient evidence to support a diagnosis of enterotoxemia, as toxin production may occur in the gut of a moribund animal suffering from other disease entities. Brain lesions in lambs of group I were characterized by focal noninflammatory necrosis, marked vascular dilatation, and small to large hemorrhages. The brains of lambs in group II had focal necrosis, neuronal degeneration, and loss of nerve fibers and neuroglial elements. An early, transitory, neutrophilic leukocyte invasion was noticed in the necrotic foci. From the 2nd or 3rd day, large faintly granular eosinophilic bodies were observed near the peripheries of the lesions, especially in the area of the internal capsule. By the 3rd day, compound granular corpuscles were seen to be invading malacic tissues, apparently arising from the walls of the intact and proliferating capillaries.

By the 10th day (group III), the malacic foci were occupied by compound granular corpuscles, and the eosinophilic bodies had disappeared. The work of Hartley suggests a possible relationship of FSE to enterotoxemia.

Materials and Methods

Standard Toxin.—The standard toxin used in the experimental studies was a partially purified and dried prototoxin * prepared from filtrates of broth cultures of *Cl. perfringens* type D. The

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* Supplied by Corn States Laboratories, Omaha, Neb.

toxin was precipitated by saturating the filtrate with ammonium sulfate. The precipitated toxin was removed by filtration, resuspended in a phosphate buffer, and dried by lyophilization.

The desiccated prototoxin was stored in an evacuated desiccator over calcium chloride and refrigerated at 5 C. After removing it from the desiccator, a minimum of 50 mg. was weighed, and the remainder returned to the desiccator. A diluent of 1.0% peptone water, containing 0.25% trypsin (Difco) 1:250, was added to a final concentration of 1 mg. of toxin per milliliter. The mixture was incubated at 37 C. for 45 minutes to permit enzyme activation of the toxin, then stored in 1-, 2-, and 3-ml. quantities in rubber-stoppered vials at −20 C. No loss of potency was encountered in the toxin for up to 6 weeks.

White Swiss mice, weighing 15 to 20 Gm., were used as test animals in determining toxin potency. The mouse LD_{100} was determined by injecting various quantities of activated toxin intravenously into mice, the LD_{100} being the smallest amount of toxin killing 4 of 5 mice within 24 hours.

First attempts to determine the approximate MLD for lambs produced such variable results that it was impossible to establish a toxic dose. Type D antitoxin serum levels had not been determined, as it was believed that young lambs would not have demonstrable titers. This, however, was found to be an erroneous supposition. Thereafter, the type D antitoxin titers of all experimental lambs were determined, and only lambs with titers negative at the ¼-unit level were used for experimentation. The antitoxin titers of 76 lambs will be reported later.[8]

Precalculated doses of trypsin-activated toxin were pipetted into bottles containing 50 ml. of sterile peptone water. Toxin was injected into the jugular vein by slow continuous drip through 50 intramedic polyethylene tubing (internal diameter of 0.023 inch). Twenty-five to 30 minutes was required for perfusion of the toxin inoculum. The time interval between completion of toxin inoculation and death of each lamb was recorded.

In the process of titrating lamb serum for type D antitoxin, it was observed that some mice inoculated with Lo doses of toxin at ¼ unit of antitoxin had central nervous system disturbance. (The Lo toxin dose for the system under study was arbitrarily designated as the largest amount of toxin which, when mixed with a given unit of antitoxin and permitted to incubate for 45 minutes, resulted in death of not more than 20% of the test mice.) In order to determine if the reaction could be reproduced, mice in several groups were given injections of an inoculum consisting of ¼ unit of antitoxin plus a Lo dose of toxin. Surviving mice were kept for a minimum of 72 hours, and those with clinical signs of a CNS disturbance were retained for further examination.

The question arose as to whether the CNS disturbance resulted from the action of the combined toxin and antitoxin, or from a critical dose of toxin. Several mice were therefore given doses of toxin ranging on both sides of the MLD. Affected

TABLE 1—Toxicity of Lot 1 Clostridium perfringens Type D Toxin for Lambs

Lamb No.	Body weight (lb.)	Daily dose of toxin (μg./lb.) Day 1	Day 2	Day 3	Day 4	Day 5	Hours till death
1	47	872.0	.....	.....	.....	.....	Immediate
C-967	61	81.9	.....	.....	.....	.....	1.5
C-1034	....	1.0 mg.	.....	.....	.....	.....	2.0
C-1044	65	15.3	.....	.....	.....	.....	1.5
C-1155	56	4.46	.....	.....	.....	.....	5.0
C-1176	67	1.49	1.49	4.9	.....	.....	6.3
C-1207	53	2.82	3.3	3.7	.....	.....	5.5
8*	66	3.0	.....	.....	.....	.....	Survived
C-1293	87	2.87	3.29	3.7	4.3	5.7	6.0
C-1305	71	2.8	3.16	4.2	4.9	.....	6.0
7P1274**	64	4.6	4.6	7.8	15.6	.....	Survived
8P118**	58	6.7	34.4	77.5	.....	.....	Survived
8P411**	78	4.4	5.7	12.8	.....	.....	7.5
8P445**	84	11.9	14.8	20.8	29.7	41.6	3.5

* Only 1 dose was administered to determine if delayed subacute intoxication might develop.
** Lambs inoculated with lot I toxin after it had been removed from refrigerator.

TABLE 2—Titration of Toxicity of Lot II Clostridium perfringens Type D Toxin for Lambs

Lamb No.	Body weight (lb.)	Daily dose of toxin (μg./lb.) Day 1	Day 2	Day 3	Hours till death
8P532	68	14.7	44.1	.....	2.5
8P530	45	11.1	.....	.....	2.2
8P583	84	5.9	8.3	23.8	1.5
8P590	70	10.7	21.4	28.5	12.0
8P588	95	15.7	.....	.....	3.0
8P620	74	16.8	.....	.....	2.25
8P648	97	8.2	10.3	.....	3.0
8P708	83	9.0	9.5	10.8	3.3
8P702	91	9.0	.....	.....	6.2
8P745	89	9.2	.....	.....	3.3
8P853	48	8.7	.....	.....	3.0

mice were killed at various intervals in order that the pathogenesis of the brain lesions might be determined.

Field Cases of Enterotoxemia.—Lambs dying of clinically diagnosed, naturally occurring enterotoxemia were necropsied as soon as possible after death. To confirm the clinical and necropsy diagnosis of enterotoxemia, contents of the small intestine and cecum were collected and tested for the presence of type D toxin. Direct smears of the intestinal contents were Gram-stained and examined for large, gram-positive bacilli morprologically resembling *Cl. perfringens.* If such organisms were present in relatively large numbers, the intestinal contents were centrifuged for 30 to 40 minutes at 10,000 r.p.m. and the supernatant fluid, in doses of 0.2 to 0.4 ml., was injected intravenously into 3 test mice. If toxin was present, an attempt was made to identify the toxin by serum neutralization. Type-specific *Cl. perfringens* typing serums * were used to identify the toxin.

Preparation of Tissues.—Following death, the brains of all lambs and mice were immediately removed from the calvarium and fixed in 10% buffered formalin. After fixation, each brain was subdivided into transverse sections approximately 1 cm. thick and examined for gross lesions. Paraffin sections of the lamb brains were prepared from the following areas: cerebral cortex at the coronary and calcarine fissures, caudate nuclei, basal ganglia, thalamus, hypothalamus, corpora quadrigemina, pons, brachium pontis, cerebellum, and medulla. Tissue sections were routinely stained by Nocht's method and frequently with hematoxylin and eosin or cresyl echt violet. In some instances, sections were stained by the methods of Weil, Holzer, Marchi, and Cajal (gold sublimate).

TABLE 3—Response of White Mice to Intravenous Graded Doses of Lot II Clostridium perfringens Type D Toxin

Toxin dose (mg.)	No. of mice			
	Injected	Survived	Dead	CNS signs
0.0005	3	3	0	0
0.0006	6	5	1	0
0.0007	6	5	0	1
0.0008	6	3	2	1
0.0009	6	3	2	1
0.001	11	5	4	2
0.00115	5	0	5	0
0.00125	5	1	3	1
0.00135	5	0	5	0
0.00150	6	1	5	0
0.00153	5	0	5	0
0.002	3	0	3	0
Total	67	26	35	6
Per cent	....	38.8	52.2	8.9

* Obtained from Burroughs Wellcome and Co., London, England.

TABLE 4—Experimental Data and Treatment of 18 Lambs Killed by Injection of One Lethal Dose of Clostridium perfringens Type D Toxin

Lamb No.	Body weight (lb.)	Toxin (μg./lb.)	Hours till death
C-967	61	81.9	1.5
C-1034	....	1.0 mg.	2.0
C-1044	65	15.3	1.5
8P530	45	11.1	2.2
8P588	95	15.7	3.0
8P829	49	8.9	6.0
8P832*	57	9.1	6.0
8P836*	66	9.1	3.5
8P853*	48	8.7	3.0
8P858*	74	8.9	3.0
8P896*	46	8.9	4.5
8P865*	42	8.8	4.25
8P866*	35	8.8	4.5
8P620*	74	16.8	2.25
8P668*	75	9.3	3.5
8P702*	91	9.0	6.2
8P745*	89	9.2	3.3

* Lambs also were given radioactive iodinated blood serum albumin for plasma transudate studies.

RESULTS

EFFECT OF TYPE D TOXIN ON LAMBS

Two lots of toxin were employed and, after several trials using various amounts of lot I toxin, it was found that an intravenous dose of 3.7 to 5.7 μg. of trypsin-activated toxin per pound of body weight resulted in death of some of the lambs in 2 to 7 hours (Table 1). A sudden and marked decrease in potency of lot I toxin was noticed following an interval of time during which the toxin was not under refrigeration, and it was therefore discarded.

The second lot of toxin required 8.7 to 9.3 μg. of trypsin-activated toxin per pound of body weight to kill experimental lambs in 2 to 12 hours (Table 2). The mouse MLD for this lot of toxin was found to range between 0.8 and 1.0 μg. The results of the mouse MLD determination are summarized (Table 3).

Brains of 62 lambs, examined for gross and microscopic lesions, were from (1) 18 lambs given 1 intravenous lethal dose (Table 4) of type D toxin; (2) 13 lambs given 2 or more intravenous injections (Table 5) of type D toxin; (3) 19 lambs with field cases of type D enterotoxemia; and (4) 12 normal control lambs given an intravenous injection of peptone-water-trypsin diluent.

On the basis of clinical signs and gross and histopathologic lesions, the 50 experimental lambs were subclassified as having

TABLE 5—Treatment and Experimental Data for 13 Lambs Given 2 or More Intravenous Injections of Trypsin-Activated Clostridium perfringens Type D Toxin

Lamb No.	Body weight (lb.)	Daily toxin dose (μg./lb.) Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Hours till death*	Pathologic results
C-1176	67	1.49	1.49	4.9	.....	.....	.....	6.3	Acute
C-1207	53	2.28	3.3	3.7	.....	.....	.....	5.5	Subacute
C-1293	87	2.87	3.29	3.7	4.3	5.7	.....	6.0	Subacute
C-1305	71	2.8	3.16	4.2	4.9	.....	.....	6.0	Subacute
7P1274	64	4.6	4.6	7.8	15.6	.....	.....	....	Survived
8P118	58	6.7	34.4	77.5	.....	.....	.....	....	Survived
8P411	78	4.4	5.7	12.8	.....	.....	.....	7.5	Subacute
8P445	84	11.9	14.8	20.8	29.7	41.6	119.0	3.5	Acute
8P532**	68	14.7	44.1	.....	.....	.....	.....	2.5	Acute
8P583**	84	5.9	8.3	23.8	.....	.....	.....	1.5	Acute
8P590**	70	10.7	21.4	28.5	.....	.....	.....	12.0	Subacute
8P648**	97	8.2	10.3	.....	.....	.....	.....	3.0	Acute
8P708**	83	9.0	9.5	10.8	.....	.....	.....	3.3	Acute

* After last dose of toxin. ** Inoculated with lot 11 toxin.

acute (42) and subacute (8) type D intoxication.

Signs of Intoxication.—Acute.—The time interval between completion of toxin injection and onset of signs of intoxication varied from none to 4 hours, depending on the dose of toxin. The period between onset of clinical signs of intoxication and death varied from 30 minutes to 4 hours. The initial stage of intoxication was characterized by depression, dullness, yawning facial movements, and decreased interest in feed and surrounding activities. Affected lambs became increasingly restless and walked aimlessly. The gait became incoordinated, or the lamb assumed a wide base stance and had difficulty in maintaining equilibrium. Some lambs, with feet widely spread, leaned forward and pressed their heads against fixed objects. Vision seemed to be impaired; however, pathologic changes in the eyes were not observed. Lateral decubitus with the head turned to the flank was noticed, and respiration became accelerated, shallow, and irregular. Convulsive seizures, characterized by violent running movements, grinding of the teeth, champing of the jaws, excessive salivation, nystagmus, twitching of muscles, and pronounced opisthotonos, were common to most lambs almost to the time of death; a few lambs, however, developed a syndrome of recumbency, coma, and quiet death.

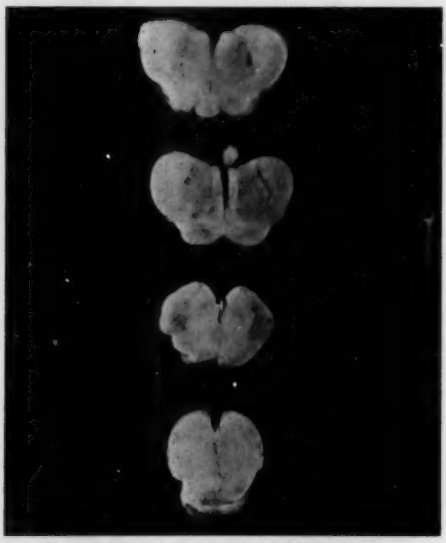

Fig. 1—Liquefaction necrosis in diencephalon and mesencephalon of lamb with experimental subacute type D intoxication.

Subacute.—Clinical signs of subacute enterotoxemia, as observed in 8 lambs, were characterized by dullness, depression, impaired vision, incoordination, aimless walking, and diarrhea. One lamb stood for considerable periods pressing its head into the corner of the pen. The duration of signs was longer in lambs with subacute cases; 1 experimentally infected lamb had signs of intoxication for approximately 11 hours. In 2 lambs with field cases, there was diarrhea, depression, blindness, and incoordination for 36 to 48 hours; terminally, there was prostration with the head in opisthotonos. Convulsive seizures were infrequently observed, but idle paddling movements of the limbs were common.

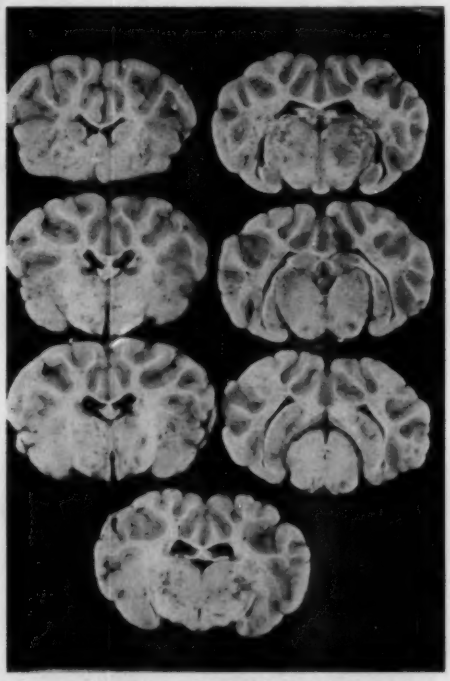

Fig. 2—Frontal sections of brain from a sheep with a field case of subacute enterotoxemia. The bilateral symmetrical distribution of focal necrosis is shown in the basal ganglia, internal capsule, thalamus, and substantia nigra (57-241).

Necropsy Findings.—Acute.—Necropsy of lambs dead from acute type D enterotoxemia revealed few gross alterations. The lesions were characterized by pulmonary congestion and edema, pronounced increase in pericardial fluid, and petechiasis of thymus, epicardium, and endocardium. Gross encephalic lesions were not discernible.

Subacute.—Multiple malacic foci were evident on gross examination of the encephalons of 3 lambs (C-241, C-1207, 8P-1387) with subacute intoxication. Bilateral symmetrical lesions were seen in the internal capsule, corpus striatum, putamen, thalamus, subcortical white matter, substantia nigra, hippocampus, and cerebellar peduncles (Fig. 1, 2).

Histopathology.—Acute.—Microscopic examination of the brains of 42 lambs with acute type D toxemia, when compared with similar sections from 12 controls, disclosed changes characterized by pronounced perivascular and intercellular edema. Considerable variation was observed in the degree and distribution of plasma effusion. The perivascular spaces of Virchow-Robin and His were dilatated and frequently contained homogeneous, eosinophilic plasma. The glial processes which extended from the perivascular glial membrane to the adventitia of the arterioles gave a crenated outline to the perivascular spaces (Fig. 3). Walls of the more severely affected arterioles had a homogeneous eosinophilic or hyalinized appearance, and an occasional vacuole was discernible in the wall. Swollen hypochromatic endothelial cells were occasionally observed lining the vessels.

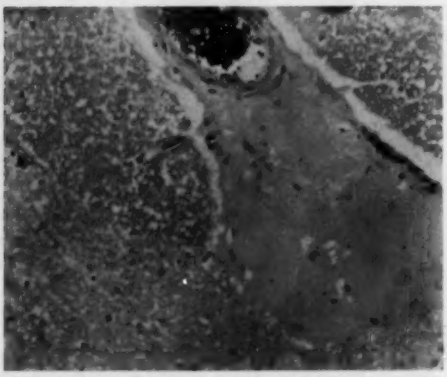

Fig. 3—Perivascular and interstitial edema in the internal capsule of a lamb dead from naturally occurring enterotoxemia (7P1304-2). Nocht's stain; x 102.

The subarachnoid spaces were distended and the pia mater was separated from the cortex by an appreciable amount of fluid. Effusion of plasma into the perivascular spaces commonly led to intercellular edema, as seen in the cribriform spongy appearance of the brain substance and in the prominently distended pericellular spaces. The intercellular edema gave a "moth-eaten" appearance to some nerve tracts (Fig. 4, 5) and was more pronounced in the white tracts of the basal ganglia, thalamus, and cerebellar peduncles. Sections stained by Marchi's method for degenerating myelin did not have evidence of pathologic alterations in the myelin. Examination of the cerebellums of a few lambs

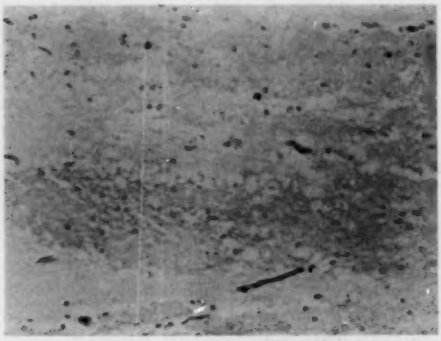

Fig. 4—Brain from sheep with experimental acute intoxication, showing edema in the fiber tracts of the caudate nuclei. Notice the rarefied or spongy character of the tissue. Neuroglia are swollen and hypochromatic (8P648). Nocht's stain; x 93.

revealed perivascular and intercellular edema, especially in the Purkinje cell stratum.

Study of sections of the cerebellum, cerebrum, basal ganglia, thalamus, corpora quadrigemina, and pons revealed focal to diffuse areas of degeneration and necrosis. The anatomical location of such foci closely agreed with the sites of the gross lesions seen in lambs with subacute field cases. The degrees of ganglion cell alteration appeared to vary with the time between injection of toxin and death of the lamb. The ganglion cells adjacent to the perivascular accumulations of plasma were acutely swollen, had large vesiculated nuclei, a perinuclear pallor, and fine ectoplasmic reticulation. In general, the swollen ganglion cells were hypochromatic, and the perikaryon contours were fuzzy and indistinct. Chromatolysis with rupture and effusion of cytoplasm into the intercellular spaces was observed, and such cells appeared as ghost or shadow forms. Occasional small clusters of neurons had cellular changes characterized by atrophy of the cell and its processes, basophilic hyperchromatism, partial to complete loss of cytoplasmic detail, and homogeneity of the nuclei. Ganglion cells of the corpus striatum and thalamus were usually more severely affected than those in the cerebral cortex; such changes were less common in the corpora quadrigemina and pons.

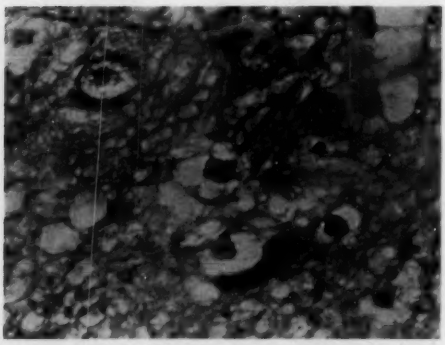

Fig. 5—Acute experimental enterotoxemia in a lamb. Section of thalamus showing perivascular and pericellular edema. The ganglion cells are acutely swollen and the cellular outlines are poorly defined (7P1304-8). Nocht's stain; x 405.

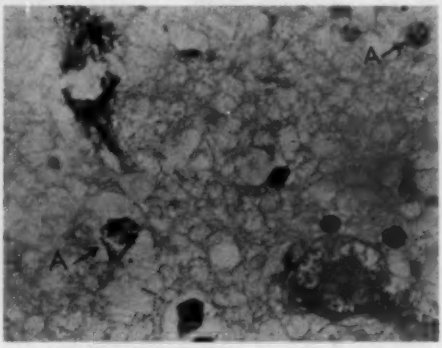

Fig. 6—Subacute experimental enterotoxemia in a lamb. The neurons are swollen and vacuolated, the nuclei show lysis and rhexis. (A) degenerating astrocytes (8P411-2). Nocht's stain; x 373.

Study of sections from the cerebellum of lambs dying within 2 hours after toxin injection disclosed little evidence of cellular disease, while study of those from lambs surviving longer revealed a variety of changes in the Purkinje cells. Individual cells were acutely swollen and presented a pale-staining, reticulated or finely granular cytoplasm and karyoklasis of the nuclei. Focal absence of Purkinje cells, or ghost forms devoid of nuclei, were also noticed. Atrophic, crenated, hyperchromatic Purkinje cells were less frequently observed.

Of the neuroglial cells, the astrocytes appeared to be the more severely damaged.

Clasmatodendrosis was evident in some sections prepared by Cajal's gold sublimate method; however, study of the sections stained by Nocht's, Holzer's, and cresyl echt violet methods revealed acutely swollen, pale, granular or fragmented glial nuclei.

Subacute.—Histopathologic lesions in lambs with subacute intoxication were of the same general character as in those with the acute form, but were more severe and further advanced. The substance of the brain was hypochromatic, rarefied, and had a spongy or vacuolated appearance (Fig. 6). Perivascular edema was pronounced, the capillary walls appeared to be hyalinized, and the endothelial cells were swollen and hypochromatic (Fig. 7). Perivascular spaces were distended with plasma, and in some instances degenerative changes occurring in the basal ganglia, thalamus, corpora quadrigemina, and cerebellum were suggestive of early malacia. The nerve tracts were hypochromatic, poorly defined, and had a "moth-eaten" appearance (Fig. 8, 9). Occasional clusters of small, round, homogeneous, pink-staining bodies were noticed immediately adjacent to damaged nerve tracts.

In sections from the brain of lamb C-1207, there were focal areas of liquefaction necrosis. Necrotic and malacic foci occurred in both white and gray matter,

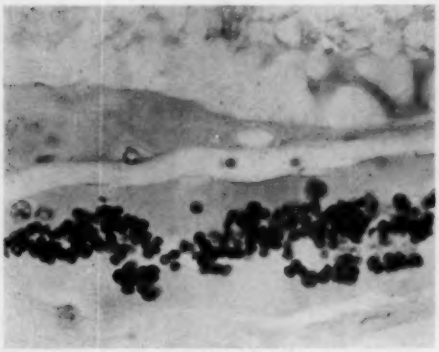

Fig. 7—Section through diencephalon of lamb dead of subacute experimental enterotoxemia. There is a pronounced perivascular edema, and the endothelial cells are swollen and hypochromatic (8P411-1). Nocht's stain; x 405.

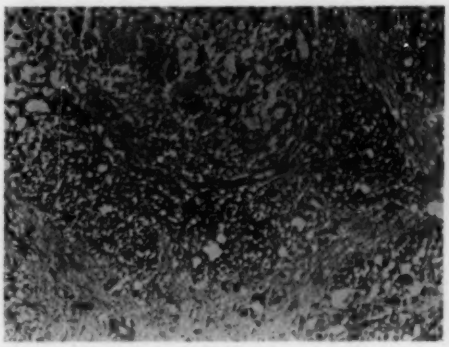

Fig. 9—Higher magnification of Figure 8. (7P1293-8). Nocht's stain; x 93.

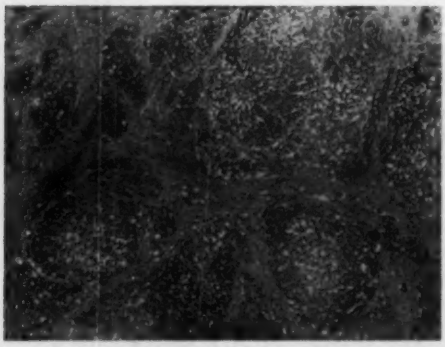

Fig. 8—Brain from sheep with subacute experimental enterotoxemia. Section of internal capsule shows edema and necrosis of fiber tracts (7P1293-8). Nocht's stain; x 37.

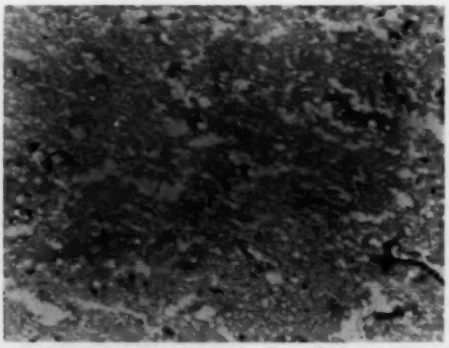

Fig. 10—Section through corpora quadrigemina showing liquefaction necrosis in subacute experimental enterotoxemia. (7P1207-14). Nocht's stain; x 93.

but were more pronounced in the white tracts of the corpus striatum, internal capsule, and thalamus (Fig. 10). In some instances, there was evidence of hypertrophy and hyperplasia of the capillary endothelium in or adjacent to the necrotic tissue. An occasional polymorphonuclear leukocyte was noticed; however, the lesions were of a noninflammatory, liquefactive nature.

TABLE 6—Treatment of Mice Having Central Nervous System Disturbances

Treatment of mice	No. of mice	
	Treated	CNS signs
Type D toxin LD_{50} determinations	67	6
Lamb serum antitoxin titrations:		
0.1 unit	40	8
0.25 unit	102	12
0.5 unit	30	6
Total	239	32*
Per cent	.....	12.9

* 24 of the 32 were selected for histopathologic study.

The development of neuronal disease was accompanied by a progressive degeneration of the astrocytes. Changes in the glia were characterized by poor staining quality and acute swelling of the nuclei. Macroglial nuclei were weakly basophilic or amphophilic and, in more advanced stages, appeared as pale, pink, indistinct elements. Fragmentation or disappearance of glial cells occurred in areas of necrosis. While rod forms of the microglia appeared to be increased in the necrotic tissue, compound granular corpuscles were not seen.

EFFECT OF TYPE D TOXIN ON MICE

The experimental data pertaining to the effect of Type D toxin on mice are given (Table 6).

Clinical Signs.—Duration of signs was variable; some mice recovered within 24 hours, others died within the same period of time, and others had clinical signs for several days. Neurologic signs of intoxication varied with the individual mouse, the more constant signs being hyperaesthesia, chorea, opisthotonos, torticollis, incoordination, circling, convulsions, and rolling. The majority of the mice had impaired equilibrium, and feeding became increasingly difficult.

Histopathology.—A marked consistensy in the symmetrical location of lesions was noticed; however, the extent and degree of severity of the lesions was not always of equal bilateral proportions. Considerable consistency was observed in the anatomical location of lesions in the encephalons of the 24 mice (Fig. 11). Lesions were more commonly observed in the basal ganglia, thalamus, corpus callosum, medial and lateral geniculate bodies, reticular substance, brachium pontis, vestibular nuclei and tracts, and granular layers of the cerebellum. Lesions were also observed in the substantia nigra, red nucleus, medial lemniscus, roof nuclei of the IV ventricles, corpus medulare cerebelli, genu of 7th cranial

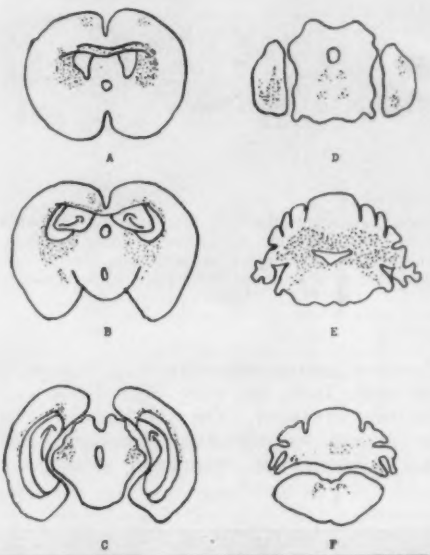

Fig. 11—Diagrammatic sketch of frontal sections of mouse brain, showing anatomical distribution of lesions (mouse 8). (A) Level of caudate nuclei; (B) level of optic chiasma; (C) section through medial geniculate body; (D) section through posterior colliculi; (E) pons and cerebellum at level of VIII nerve; and (F) section through cerebellum and medulla.

nerve, and cerebral cortex. The histopathologic changes resulting from type D intoxication are described in chronologic order:

At 7 Hours.—Lesions, consisting of perivascular and intercellular edema and acute neuronal disease, were minimal in brain sections of the 2 mice (M26 and M27)

killed 7 hours after injection of a test dose of toxin (Fig. 12). Perivascular spaces were distended but contained little plasma protein. Focally, the brain substance appeared rarefied and vacuolated. Occasional groups of ganglion cells had large vesiculated nuclei, poor in chromatin, and perinuclear pallor, ectoplasmic reticulation, and irregular or poorly defined cellular

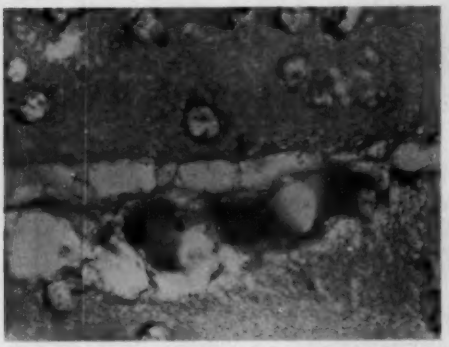

Fig. 12—Section through anterior central gray nuclei of mouse 26, killed 7 hours after injection of type D toxin. Notice perivascular edema, intercellular edema, and acutely swollen ganglion cells (M26-8). Nocht's stain; x 373.

contours. These lesions were more pronounced in the brain substance adjacent to the lateral ventricles.

At 18 to 24 Hours.—The brains of 3 mice (M1, M2, and M3) were the first mouse tissues collected and serial sections were not prepared; however, sections were cut from the cerebral hemispheres, basal ganglia, hippocampus, corpora quadrigemina, pons, and cerebellum.

Multiple early malacic lesions were present in the cerebral cortex, basal ganglia, corpus callosum, cerebellar peduncles, and white matter of the cerebellar folia (Fig. 13); however, the malacic lesions had not progressed to a stage of liquefaction necrosis.

At 30 to 36 Hours.—In 2 mice (M9 and M28) killed at 30 to 36 hours postinoculation, perivascular and pericellular edema was marked and somewhat generalized. Many of the lesions, particularly in the basal ganglia and thalamus, had progressed to a stage of liquefaction necrosis. Three developmental stages were seen in a few of the lesions: an inner zone of liquefac-

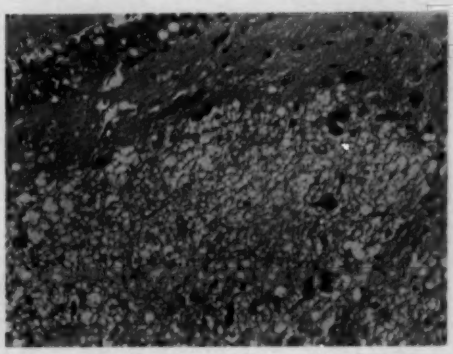

Fig. 13—Cerebellum of mouse killed 24 hours postinoculation, showing necrosis in roof nuclei (M-1). Nocht's stain; x 93.

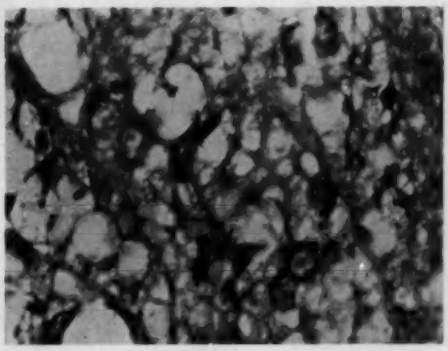

Fig. 14—Section of thalamus of mouse killed 30 hours after injection of type D toxin. There is interstitial edema and ganglion cell degeneration (M-9-15). Nocht's stain; x 373.

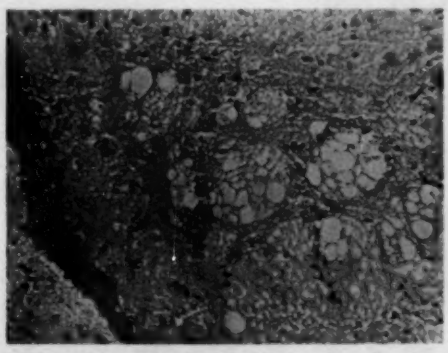

Fig. 15—Necrosis in basal ganglia of mouse killed 48 hours after injection of toxin. Notice the liquefaction necrosis in the fiber tracts (M-5). Nocht's stain; x 93.

Fig. 16—Liquefaction necrosis in area of vestibular and acoustic nuclei of mouse killed 3 days post-inoculation (M-8-42). Nocht's stain; x 37.

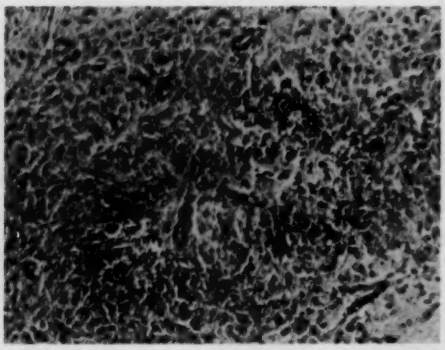

Fig. 17—Section from thalamus of mouse killed 4 days after injection of toxin, illustrating glial proliferation in an old focus of liquefaction necrosis. A small central zone of necrosis remains (M-14-10). Nocht's stain; x 93.

tion necrosis, an intermediate area of malacia, and an outer irregular zone characterized by acute neuronal degeneration and edema (Fig. 14). The necrotic and malacic lesions contained extravasated erythrocytes, a few persistent and intact capillaries, cellular debris, and viable-appearing microglia. Neutrophils were not discernible. Small malacic lesions and clusters of atrophic hyperchromatic ganglion cells were observed in the cerebral cortex.

Degenerated, swollen, pink-staining macroglia were observed in the corpus callosum. Vacuolation, necrosis, and hemorrhage were characteristic changes occurring in the cerebellar peduncles and pons. Foci of pyknotic cells were observed in the granular layer of the cerebellum; a few Purkinje cells were acidophilic and the cytoplasm appeared to be coagulated.

Second Day.—There was liquefaction necrosis in most lesions; however, little change in the character and distribution of lesions was observed in the brains of the 3 mice (M4, M5, and M10) killed 48 hours following toxin injection (Fig. 15). Elongated hyperchromatic capillary endothelium, cellular debris, and a few microglia were present in the necrotic tissue. Most of the ganglion cells in the tissue adjacent to the necrotic foci were atrophic and hyperchromatic, and contained only a narrow zone of acidophilic cytoplasm. A focal increase in microglia was observed in the subependymal tissue near the dorsal angle of the lateral ventricle. Astrocytes in the tissue near the foci of malacia appeared to have acutely swollen nuclei.

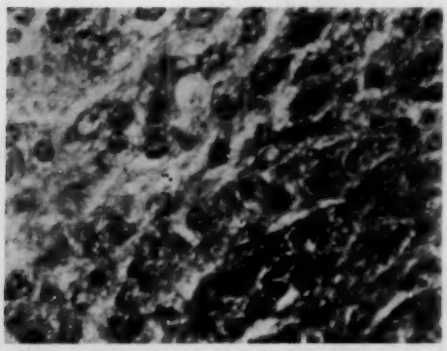

Fig. 18—Higher magnification of Figure 17, showing mitotic figure in glial cell (M-14-10). Nocht's stain; x 405.

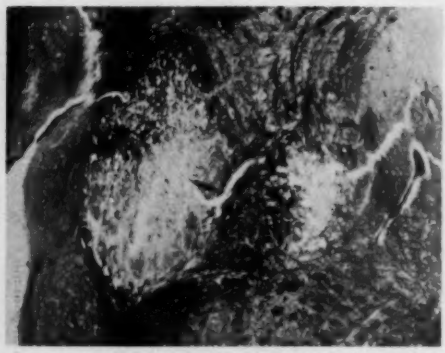

Fig. 19—Necrosis of acoustic and vestibular nuclei and tracts in mouse killed 4 days after inoculation of toxin (M-11-19). Weil's stain; x 37.

Third Day.—Liquefaction necrosis was observed in brain sections of the 6 mice (M6, M7, M8, M20, M21, and M22) killed 3 days after injection of toxin (Fig. 16). A proliferation of round, intensely basophilic cells was noticed in and surrounding a few capillaries in the brain of a mouse (M8); however, compound granular corpuscles were not observed.

Fourth Day.—In sections of the encephalons of 3 mice (M11, M12, and M14), collected on the 4th day following toxin injection, there was pronounced increase in macroglia in and adjacent to areas of necrosis in the basal ganglia, thalamus and corpus callosum (Fig. 17). The smaller lesions were recognizable only by the mass of proliferating glial cells which were replacing the previously necrotic tissue. A marked variation was noticed in the size, shape, and staining reaction of the glial cells. The majority were large, spindle-

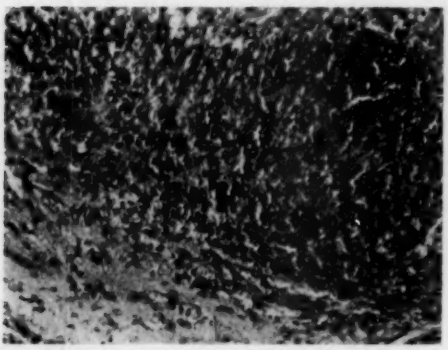

Fig. 20—Necrotic lesion in the thalamus of mouse showing glial proliferation. Mouse killed 7 days after injection of toxin (M-16-10). Nocht's stain; x 93.

shaped or irregularly stellate, deeply basophilic cells, some of which contained mitotic figures (Fig. 18). The latter cells, when stained by Holzer's method, had a dense, shrubby appearance, suggestive of astrocytes. The large lesions contained central areas of liquefaction necrosis surrounded by a wall of astrocytes. Capillaries had invaded the lesions and, in some sections, a mesh of capillaries had been formed (Fig. 19).

Fifth, Sixth, and Seventh Days.—The lesions observed in the brains of mice killed at 5 (M23 and M24), 6 (M15), and 7 (M16 and M13) days following toxin injections were so similar that they have been grouped together for discussion. The lesions were characterized by capillary invasion and astrocyte infiltration into the necrotic tissue (Fig. 20). Mitotic figures were discernible in some cells. Maturation and differentiation of the glial cells was the only discernible change between the 5- and 7-day-old lesions. Healing was not complete in any of the sections (Fig. 21).

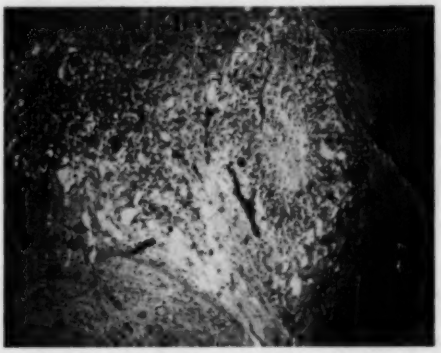

Fig. 21—Liquefaction necrosis and glial scar formation in vestibular nuclei of mouse killed 7 days after administration of toxin (M-15-30). Nocht's stain; x 37.

A study of the lesions occurring in mouse brains following injection of type D toxin revealed an absence of compound granular corpuscles. At no stage of the development of the neurologic lesions were such cells recognized.

Discussion

Clinicopathologic Correlations.—According to Holmes,[10] interpretation of neurologic signs may be complicated by the capacity of the nervous system to adapt itself to changes in conditions imposed on it by disease. The acute onset and short duration of ovine enterotoxemia, and the diffuse edematous changes occurring in the basal ganglia, internal capsule, thalamus, pons, and cerebellum, renders the correlation of lesions and neurologic signs difficult.

Progressive drowsiness, confusion, impairment of vision, and generalized convulsions are, according to Scheinker,[14] neurologic manifestations probably related to

increase in bulk of the brain with consequent increase in intracranial pressure. Such clinical signs may be related to increase in brain bulk due to edema, and it is therefore possible that these signs, all of which are observed in acute enterotoxemia, are related to intracranial accumulation of plasma transudate.

Nonspecific sensory impulses necessary for alerting all areas of the cerebral cortex may be diffusely projected from the thalamus to the cerebrum by way of the internal capsule in connection with the projection of visual, auditory, and general sensory impulses to specific sensory areas.[2] The pronounced edema involving the basal ganglia, internal capsule, and thalamic area probably interferes with transmission of sensory impulses to the cerebral cortex, and thus may account for the early manifestation of depression and dullness by most lambs affected with enterotoxemia. Perivascular and intercellular edema occurring in the hypothalamus of some affected lambs may also interfere with the capacity of the animal to regulate the body temperature. This may account for the subnormal body temperatures observed in animals with enterotoxemia.

Nystagmus, a neurologic sign observed in lambs prior to and during convulsive seizure, is probably related to edema and degenerative lesions in the cerebellum and cerebellar peduncles, especially in the area of vestibular nuclei and tracts. The only afflictions of the central nervous system which produce nystagmus are those which involve the central end stations of the vestibular nerves or their immediate connections.[10] Nystagmus occurs, therefore, with disease of the pons, midbrain, and cerebellum and is seen in its most characteristic form in acute lesions of the latter.

Circling, torticollis, and rolling ambulatory movements were the more pronounced signs of neurologic disease in mice. Such clinical signs of impairment of equilibrium may be correlated to lesions involving the vestibular nuclei, vestibular tracts, reticular substance, and nuclei located in the roof of the 4th ventricle.

Tremors which were frequently observed in intoxicated lambs and mice are apparently associated with lesions in the basal ganglia. The basal ganglia are directly concerned with such involuntary movements as tremors and chorea, and the production of such movements is related to interference with the cortico-subcortical circuit which lies in part in the basal ganglia.[2]

Comparison with Other Disease.—Hartley[9] reported that a disease occurring in sheep in New Zealand is characterized by focal symmetrical encephalomalacia (FSE). He examined 37 lambs which were allotted to 4 groups according to the duration of neurologic signs. The 4 lambs in group I were found dead and were unobserved prior to death. The 24 lambs in group II had a variety of neurologic manifestations for 1 to 6 days before death. Group III consisted of 8 lambs which manifested neurologic signs for 7 to 14 days. The 1 lamb in group IV had been affected for more than 2 weeks.

Macroscopic encephalic lesions were present in 3 lambs from group I, and in 27 from groups II and III. Only microscopic lesions were found in the 1 lamb from group IV. The lesions were bilaterally symmetrical and were characterized microscopically by noninflammatory parenchymal necrosis, hemorrhage, and vascular dilatation.

Hartley felt that the demonstration of *Cl. perfringens* type D toxin in 1 of the 4 lambs from group I, together with necropsy findings simulating enterotoxemia, tended to incriminate this toxin as the cause of the brain lesions. He further postulated that the brain lesions occurring in lambs from groups II, III, and IV may have resulted from sublethal doses of the same toxin.

The presence of necrotic lesions in the brains of the lambs from group I would suggest that the animals had probably been affected longer than the clinical observations indicated, since study of lambs dying from acute experimental type D intoxication revealed only edematous changes. The anatomical distribution and character of lesions in FSE resembled those observed in experimental enterotoxemia, with the exception that hemorrhages were not found in the latter and no mention of edema was made in the former. While the evidence presented by Hartley indicates that the lesions in FSE may result from type D enterotoxemia, it is not conclusive, since there are some variations in the nature of the lesions as compared to the lesions found in experimental type D intoxication. If

malacic lesions are present in the cerebral cortex of lambs with FSE, the condition might more closely resemble polioencephalomalacia as described by Jensen *et al.*[12]

Polioencephalomalacia was reported by Jensen *et al.*[12] as a noninfectious disease of pasture and feedlot cattle and sheep, characterized by multiple foci of malacia and necrosis in the cerebral cortex and, to a lesser extent, in the cerebellum and thalamus. Clinically, the disease was characterized by sudden onset, followed by an acute or chronic course. The clinical signs were similar to those of acute enterotoxemia, with the exception that affected animals usually lived longer following the development of convulsive seizures. Pathologic changes were more marked in the cerebral cortex and were recognized grossly as multiple foci of malacia and necrosis. Histopathologic study of the cerebral cortex disclosed focal to diffuse neuronal degeneration and liquefaction necrosis. All attempts to detect toxins of *Cl. perfringens* in intestinal contents of animals with acute cases were unsuccessful, and *Cl. perfringens* antitoxin could not be demonstrated in serums of recovered animals. Although the cause of polioencephalomalacia is unknown, the clinical and pathologic manifestations are suggestive of intoxication or possibly anoxia. Polioencephalomalacia might readily be confused with subacute type D enterotoxemia but can be differentiated by examination of the brain.

In recent years, other acute softenings in the central nervous system of animals have been described. Innes and Plowright[11] recorded a poliomyelomalacia in lambs which was thought to have resulted from a copper deficiency. Cordy[5] described a focal symmetrical encephalomalacia involving the globus pallidus and substantia nigra of horses in California. The condition was experimentally shown to be related to consumption of large quantities of a species of thistle over an extended period of time. The mechanisms producing the lesions, however, were not determined.

Carbon monoxide poisoning, cyanide poisoning, and nitrous oxide anesthetic accidents, according to Courville,[6] produce lesions in the human brain which have some resemblance to the lesions observed in experimental type D enterotoxemia in lambs. The main difference between these intoxications in man and enterotoxemia in lambs is the absence of hemorrhagic and cortical lesions in enterotoxemia.

SUMMARY

Acute and subacute forms of *Clostridium perfringens* type D intoxication were observed in animals with natural and experimental cases of enterotoxemia. Gross lesions were not discernible in the brains of lambs dying from acute enterotoxemia. Microscopic foci of softening or liquefaction necrosis were found in the basal ganglia, thalamus, internal capsule, substantia nigra, subcortical white matter, and cerebellum of most lambs affected with acute type D intoxication. The lesions were characterized by vascular congestion, degeneration of endothelium and walls of the vessels, pronounced perivascular edema, and varying degrees of intercellular edema. Pathologic changes in the neurons and neuroglia appeared to be related to the increase in plasma transudate. The anatomical sites of lesions in acute intoxication compared favorably with those observed grossly in subacute enterotoxemia.

White Swiss mice were found to be of considerable value in a study of the neurotoxic effects of *Cl. perfringens* type D toxin. The chronologic pathogenesis of encephalic lesions in type D intoxicated mice appeared to be related to an initial increase in vascular permeability followed by edema, softening, liquefaction necrosis, and healing by glial scarring.

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SUMMARIO IN INTERLINGUA

Enterotoxemia de Oves. I. Effectos de Toxina de *Clostridium perfringens* Typo D in le Cerebros de Oves e Muses

Acute e subacute formas de intoxication per *Clostridium perfringens* typo D esseva observate in animales con enterotoxemia natural e experimental. Lesiones macroscopic non esseva discernibile in le cerebros de agnos morte ab enterotoxemia acute. Focos microscopic de necrosis mollificante o liquefactori esseva trovate in le gangliones basal, le thalamo, le capsula interne, le substantia nigre, le substantia blanc subcortical, e le cerebello del majoritate del agnos afficite de acute intoxication typo D. Le lesiones esseva characterisate per congestion vascular, degeneration endothelial e parietal in le vasos, pronunciate grados de edema perivascular, e varie grados de edema intercellular. Alterationes pathologic in le neurones e le neuroglia pareva esser relationate con le augmento de transsudato plasmatic. Le sitos anatomic de lesiones in acute intoxication correspondeva ben a illos observate macroscopicamente in enterotoxemia subacute.

Esseva trovate que muses switze blanc esseva de considerabile utilitate in le studio del effectos neurotoxic de toxina de *Cl. perfringens* typo D. Le chronologia del pathogenese de lesiones encephalic in muses a intoxication per typo D pareva esser relationate con un augmento initial in le permeabilitate vascular, sequite per edema, mollification, necrosis de liquefaction, e reformation per cicatrisation glial.

Enterotoxemia of Sheep. II. Distribution of I^{131} Radioiodinated Serum Albumin in Brains of *Clostridium perfringens* Type D Intoxicated Lambs

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THE NEUROPATHOLOGIC effect of *Clostridium perfringens* type D toxin on the brains of lambs and mice was studied and reported in an earlier publication.[7] In that phase of the investigation, it was found that the primary encephalic lesion occurring in lambs affected with acute type D intoxication was perivascular and intercellular edema.

A method which would estimate the extravascular plasma in the encephalon would help substantiate the microscopic observation and assist in determining the true significance of the edema. Of the methods that might be used to estimate plasma transudation, it was believed that radioactive isotopes would be suitable for such quantitative determinations.

REVIEW OF LITERATURE

The application of radioactive tracer procedures to biological problems has created a new and rapidly expanding field of investigation. Radioactive agents have been found to be valuable tools in measuring physiologic processes and pathologic deviations.

Aust *et al.*,[1] using radioactive iodinated human serum albumin to determine blood volumes, found that the method was simple and rapid, requiring only one blood sample, and that calculations were minimal. It was also observed that discrepancies in blood volumes could be correlated with the anticoagulants used. More accurate results were obtained with heparin than with sodium oxalate or sodium citrate.

Radioactive iodinated human serum albumin (RISA) was used by Crispell *et al.*[4] to determine plasma volumes. Iodinated albumin was found to be chemically stable, and surface radiation patterns indicated that there was no gross differential uptake by the thyroid or liver.

Postmortem examination of infants and animals has indicated that hypoxia was frequently associated with cerebral capillary dilatation, edema, transudation of plasma, and hemorrhage. The association of hypoxia with plasma transudation was determined by Slobody *et al.*[10] by measuring the rate of RISA migration from blood to cerebral spinal fluid in dogs. Hypoxia was created by using mixtures of nitrogen and oxygen. Their experiments were designed to use paired samples. Following hypoxia in pups, the early appearance of significantly increased cerebrospinal fluid radioactivity as compared to the control value indicated that hypoxia is apparently associated with increased permeability of the blood brain barrier.

Walker and Ross[11] measured the transcapillary leakage rates of plasma albumin and globulin. Comparisons of these rates of exchange with their corresponding protein concentrations (in Gm./kg.) reveals what appears to be a barrier to leakage below a circulating albumin concentration of 0.6 Gm./kg. with a corresponding globulin value of 0.4 Gm./kg. The mechanism of this apparent barrier at low protein concentrations is not clear, but it is interesting that the barrier is relatively higher for globulin than for albumin. This would be expected, since globulin has a higher molecular weight than albumin.

In order to analyze the translocation of plasma between tissues of the mouse during experimental traumatic shock, Friedman[6] determined the plasma volumes of various tissues in the mouse by giving mice injections of radioiodinated albumin and assaying the tissue samples for radioactivity.

Rothschild *et al.*[8] studied tissue distribution of I^{131}-labeled human serum albumin following intravenous administration in 2 male patients with fatal cardiac disease. It was concluded that 4 to 5 days are required for essentially complete equilibration of the extravascular exchangeable albumin pools of skin and muscles with plasma albumin.

MATERIALS AND METHODS

A lot of 19 lambs, 8 to 14 weeks of age, were obtained for experimental studies of plasma transudation. Serum *epsilon* antitoxin titrations at ¼ unit per milliliter revealed that 16 lambs were negative at that level. These 16 lambs were grouped on the basis of body weight, and then paired at random. One animal in each pair served as an untreated control. Each lamb was given a 5-ml. injection of physiologic saline solution (PSS) containing 50 to 60 μc. of radioiodinated (I^{131})

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human serum albumin (RISA). A similar quantity of RISA was diluted to 1 liter in PSS and used as a reference standard. A blood sample was taken from the opposite jugular 10 to 12 minutes after injection of the isotope. The blood was collected in heparinized tubes, and 2 ml. of whole blood was pipetted into a rubber-stoppered test tube. The remainder of the blood was centrifuged, and 2 ml. of plasma was pipetted into a similar tube.

Immediately after injection of RISA, the principals were given, via the jugular vein, 50 ml. of a 1% peptone water, containing 9 μg. of activated toxin, per pound of body weight. Control lambs were similarly given 50 ml. of 1% peptone water. When clinical signs of central nervous system disturbance developed in the principals, a 2nd blood sample was collected from both lambs and prepared as previously described. Each lamb was then heparinized, using an intravenous dose of 1.4 mg. of heparin sodium per pound of body weight. Both lambs were exsanguinated as soon as the principal developed convulsive seizures. Blood remaining in the vessels of the brain was removed by perfusing 1.5 to 2.0 liters of buffered saline through the carotid arteries, followed by 500 ml. of 10% formalin. The brain was then removed from the calvarium, weighed, and divided longitudinally into equal halves. Four aliquots of tissue were removed and weighed from half of each brain. The 1st sample included portions of the caudate nuclei and adjacent cerebral cortex; the 2nd was taken from the area of the thalamus and adjacent cortex; the 3rd included tissue from the hippocampus and corpora quadrigemina; and the 4th was removed from the pons and cerebellum. The remaining brain tissue was fixed in 10% formalin.

The number of radiation emissions per minute was counted for each sample in a deep-well scintillator. The net counts per minute were calculated by subtracting the background radiation from the total counts per minute per sample. The radiation activity of the brain was determined by multiplying the weight of the brain (in grams) by the average number of radiation emissions per minute per gram of the measured samples of brain tissue.

Tissue distribution of I^{131}-labeled albumin was determined according to the formula of Rothschild *et al.*[5]

$$I^{131}\text{ distribution} = \frac{\text{tissue net counts/min./Gm.}}{\text{plasma* net counts/min./Gm.}}$$

It is assumed that the distribution of I^{131} in the tissue represents a valid approximation of plasma transudate in the tissue. This assumption is based on the conclusion of Friedman[6] that the plasma volume of tissue is the ratio of radioactivity per unit weight of tissue to radioactivity per unit weight of plasma. It is recognized that this method does not measure the difference in the effusion of the various plasma components into the tissue.

Radioiodinated I^{131} human serum albumin was used as the tracer vehicle because of its availability. The lambs used in this investigation were observed for as long as 6 hours after injection of RISA, and no ill effects were observed.

* Second sample.

RESULTS

Radioactive iodinated human serum albumin was used to determine the validity of the microscopic evidence of edema in the brains of lambs killed by intravenous injections of *Cl. perfringens* type D toxin, and to estimate the difference in the amounts of plasma transudate in the brains of control and intoxicated lambs. Eight pairs of randomly selected normal lambs were used; however, deposition of part of the RISA into the tissues at the site of injection in 1 or both lambs of 2 pairs necessitated their removal from the isotope studies. Therefore, only 6 pairs of lambs are included in the results (Table 1).

The results, while not giving an accurate measurement of the total volume of plasma transudate in the organ, might be used to estimate the transudate volume. The results show a calculated average of 0.14 ml. of transudate in the brain of the controls as compared to a calculated average of 8.57 ml. in the brain of intoxicated lambs (range of 0.06 to 0.20 for controls and 3.22 to 15.41 for principals).

The increase in distribution of I^{131} in the brains of lambs intoxicated with type D toxin indicates a marked increase in vascular permeability and edema in the brain. An average 61-fold increase in I^{131} was found in the brains of the intoxicated lambs when compared to the controls.

A consistent increase was observed in the total weight of brains of intoxicated lambs when compared with the paired control. The per cent increase in the weight of the brain of the principals over their paired control ranged from 4.7 to 17.6%. In no instance did the weight of the brain of a control lamb approximate or exceed that of the principal. The results obtained from the isotope studies confirm the histopathologic observation of edema in the brain of type D intoxicated lambs.

DISCUSSION

The extensive perivascular edema, increased total brain weight, increased plasma content of the brain, and edematous nature of brain lesions indicated that the toxin in some way alters the blood-brain barrier, thereby increasing permeability of the vascular structures. Increase

TABLE 1—I^{131} Distribution in the Brains of Type D-Intoxicated and Paired Control Lambs

Lamb No.	Body weight (lb.)	RISA dose (μc.)	Toxin dose (mg./lb.)	Hours until death	Brain weight (Gm.)	% Difference in weight of lamb brains	I^{131} distribution (brain)**
8P832–P*	57	55	9.1	6.0	118.6	+17.4	6.97
8P857–C	55	55	....	6.0	101.0	....	0.13
8P836–P	66	50	9.0	3.5	115.1	+ 6.6	15.41
8P837–C	58	50	....	3.5	107.9	....	0.18
8P858–P	74	55	8.9	3.0	121.4	+14.1	9.73
8P856–C	68	55	....	3.0	105.8	....	0.17
8P896–P	46	55	8.7	4.5	112.2	+ 8.3	8.07
8P897–C	45	55	....	4.5	103.6	....	0.08
8P865–P	42	50	8.8	4.25	101.2	+ 6.6	8.06
8P868–C	34	50	....	4.25	94.9	....	0.20
8P866–P	35	50	8.8	4.5	100.5	+ 4.7	3.22
8P867–C	39	50	....	4.5	96.0	....	0.08

* P=principal; C=control

** $\frac{\text{cts./min./Gm. of brain}}{\text{cts./min./ml. of plasma}}$

in vascular permeability in enterotoxemia is not restricted to the brain, but apparently occurs in other organs as seen in pulmonary edema and hydropericardium observed at necropsy. The specific site or nature of the chemical action of the toxin on the vascular elements has not been determined.

Edema may result from an imbalance between osmotic and hydrostatic forces tending to retain fluid in the intravascular compartment and those tending to move fluid into the interstitial spaces. A variety of agents may increase capillary permeability and thus permit escape of blood plasma. According to Scheinker,[9] edema of the brain is observed in acute alcoholism, carbon monoxide poisoning, cyanide poisoning, hypertension, and nitrous oxide anesthesia accidents.

It has been customary to classify hypoxia according to the mechanisms producing such a physiopathologic state. On the basis of its mechanisms, hypoxia may be classified as: (1) anemic hypoxia in which the decreased amounts of hemoglobin do not transport sufficient oxygen to meet the respiratory requirements of the tissues; (2) stagnation hypoxia which occurs as a result of decreased blood flow and slowed blood currents which do not supply sufficient oxygen to the regional tissues; (3) histotoxic hypoxia, in which some factor interferes with the utilization of oxygen by the tissues, possibly an interference with the enzyme systems of internal respiration; (4) anoxic hypoxia, which occurs when there is a reduced volume of oxygen in the inhaled air, or when obstructive pathologic conditions in the lung inhibit normal exchange of gases. Slobody [10] found that anoxic hypoxia created by nitrous oxide anesthesia in pups caused an increase in the amount of cerebrospinal fluid, apparently as the result of increased capillary permeability.

In his extensive discussion of the mechanisms and effects of cerebral anoxia, Courville [5] points out that the immediate effects of severe degrees of hypoxia are hyperemia, edema, and hemorrhage. The effects of hypoxia on the nervous system appear to be more profound, since the tissues of this system have a greater degree of sensitivity to oxygen deprivation. Nerve cells of the cerebral cortex and Purkinje cells of the cerebellum are the most sensitive of the neuronal elements. He also stresses that variations in the degree of tissue alteration in individuals is dependent upon the survival period following onset of hypoxia. The brains of human patients who succumb to the effects of hypoxia within a day or 2 have few changes other than those incident to circulatory changes, such as hemorrhage, congestion, and edema. In patients surviving for several weeks, gross alterations are seen. In his opinion, the most pronounced lesion in hypoxia is hemorrhage, particularly in the white matter of the cerebral cortex and in the basal ganglia.

It has been postulated that the pallidum is particularly susceptible to histotoxic anoxia resulting from carbon monoxide poisoning. Courville [5] describes cortical malacia as a characteristic lesion in the human brain following fatal hypoxia resulting from carbon monoxide poisoning and nitrous oxide anesthesia accidents.

According to Yant *et al.*,[12] dogs subjected to prolonged carbon monoxide asphyxia (15 hours) developed malacic lesions in the subcortical white matter. Well-defined cor-

tical lesions have been absent in animals with naturally occurring and experimental cases of enterotoxemia in this study, and gross hemorrhages were not observed in the brains of any of the animals. These observations are believed to be of importance in eliminating hypoxia as a primary cause of brain lesions in enterotoxemia. Perivascular and intercellular edema would in all probability create a local hypoxia which in turn might cause further development of lesions in the parenchyma of the brain. It is possible that the toxin in the plasma transudate may come in contact with the neurons and thus cause alterations of these cells.

The experiments of Bullen *et al.*[3] indicated that high concentrations of type D toxin over a period of several hours in the ovine intestine increased the permeability of the organ, thus aiding in absorption of toxin and production of a toxemia. It therefore seems logical that his evidence can be used to support the hypothesis that type D toxin acts on the vascular system, causing an increase in capillary permeability.

In considering the swollen, hypochromatic endothelium and the pale, acidophilic, degenerative appearance of the walls of some vessels located within the edematous lesions, it seems reasonable to believe that the toxin may be histotoxic to the cellular components of the vascular structures thereby increasing their permeability.

SUMMARY

The use of radioactive I^{131} revealed a sharp increase in the distribution of the isotope in the brains of lambs intoxicated with *Clostridium perfringens* type D toxin, indicating a marked increase in vascular permeability. The results obtained from the isotope studies confirmed the histopathologic evidence of cerebral edema. It therefore appears that the primary action of *Cl. perfringens* type D toxin is on the vascular system, causing an increase in permeability. These observations are in agreement with the findings of others[2] who reported that oral administrations of type D toxin increased the permeability of the mouse intestine. The increase in permeability of the intestine apparently permitted absorption of the toxin into the blood or lymphatics.

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SUMMARIO IN INTERLINGUA

Enterotoxemia de Oves. II. Le Distribution de Albumina Seral a Marcation con I^{131} in le Cerebros de Agnos Intoxicate per *Clostridium perfringens* Typo D

Le uso de I^{131} radioactive revelava un augmento marcate del isotopo in le cerebros de agnos intoxicate con toxina de *Clostridium perfringens* typo D, reflectente un augmento marcate del permeabilitate vascular. Le resultatos obtenite in le studios a isotopo confirmava le evidentia histopathologic de edema cerebral. Il pare, per consequente, que le action primari de toxina de *Cl. perfringens* typo D affice le systema vascular, causante un augmento del permeabilitate. Iste observationes es in congruentia con le constatationes del alteros qui ha reportate que administrationes oral de toxina del typo D augmenta le permeabilitate del intestinos de muses. In le presente experimentos le augmentate permeabilitate intestinal permitteva apparentemente le absorption del toxina ad in le sanguine o le lymphaticos.

Enterotoxemia of Sheep. III. *Clostridium perfringens* Type D Antitoxin Titers of Normal, Nonvaccinated Lambs

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In a study of the neurotoxic effects of *Clostridium perfringens* type D toxin on lambs and mice, it was noticed that several lambs were refractory to large doses of activated type D toxin. This observation suggested that clinically normal lambs may develop active antitoxin titers, probably as the result of exposure to subclinical doses of type D toxin. It therefore became apparent that blood from all experimental lambs should be checked for antitoxin titers, and that only those with negative titers should be used for enterotoxemia experiments.

Review of Literature

The theory that *Cl. perfringens* type D toxin is the etiologic agent in enterotoxemia has been questioned on the basis of the difficulty experienced in reproducing the disease by intravenous administration of filtrates of intestinal contents from animals with clinical cases of enterotoxemia. Failure to experimentally reproduce enterotoxemia may, at times, be due to the presence of antitoxin titers in otherwise normal lambs. Bullen *et al.*[1] found that 2 of 5 lambs, 3 to 15 months of age, had antitoxin titers of 0.1 unit per milliliter. Smith and Matsuoka[4] also observed that 1 lamb in a lot of 9 feedlot-aged lambs had an antitoxin titer of 4 units per milliliter.

Bullen and Batty[2] reported that 2 experimental sheep developed antitoxin levels while on pasture. One was pastured for 4 months, and study of 3 blood samples taken during the 1st month revealed negative antitoxin titers. Study of several blood samples obtained during the 4 months when the lamb was on grass revealed the development of an *epsilon* antitoxin titer of 5 to 10 units per milliliter during the 2nd month.

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Materials and Methods

Serum samples of 76 young nonvaccinated lambs, originating from 3 different sources, were titrated for type D antitoxin. One group of 22 lambs, 5 to 7 months of age, was raised at the Colorado State University Animal Disease Research Station. A 2nd lot of 35 lambs, approximately the same age, came from a range flock in Wyoming. The 3rd lot of 19 lambs, 8 to 14 weeks of age, was obtained from a ranch near Cheyenne, Wyoming.

The toxin used in titrating the type D antitoxin titers was lot II toxin, as previously described.[3] The antitoxin units mentioned herein are based on a standard desiccated antitoxin, of equine origin, supplied by the USDA, ARS, Animal Inspection and Quarantine Branch. The desiccated antitoxin, when reconstituted according to instructions, contained 350 units of type D antitoxin per milliliter (an arbitrarily assigned unitage). The antitoxin was reconstituted by dissolving it in 1% peptone water to a final dilution of 10 units per milliliter, and distributing it in 1-, 2-, and 5-ml. quantities into rubber-stoppered vials which were then stored at −20 C.

The L_o and L_+ doses of toxin for a given unit of antitoxin were determined by mixing varying quantities of trypsin-activated toxin in 1 ml. of peptone water diluent with 0.5, 0.25, and 0.1 unit per milliliter of standard antitoxin. The mixtures were incubated at room temperature for 45 minutes, and 0.2 ml. of each dilution was injected intravenously

TABLE 1—Clostridium perfringens Type D Antitoxin Titers of 3 Lots of Nonvaccinated Lambs

Lot No.	No. of lambs	Units of antitoxin per milliliter		
		0.1	0.25	0.5
I	35			
	Number tested	13*	35	35
	Number positive	4	11**	9
II	19			
	Number tested	0	19	0
	Number positive	0	3	0
III	22			
	Number tested	9†	22	12‡
	Number positive	0	7	0
Total No. tested		22	76	47
Total No. positive		4	21	9
Per cent of positive titers		18.1%	27.6%	19.1%

* All 13 serum samples were negative at ½ and ¼ unit per milliliter.
** Includes the 9 positive at ½ unit per milliliter.
† All were negative at ¼ unit per milliliter.
‡ Includes the 7 positive at ¼ unit per milliliter.

into 5 white mice. The results of the test were read 24 hours postinoculation.

The L_o toxin dose for the system under study was arbitrarily designated as the largest amount of toxin which, when mixed with a given unitage of antitoxin and permitted to incubate for 45 minutes, resulted in death of not more than 20% of the mice. The L_+ dose of toxin was designated as the smallest amount of toxin which, when incubated with a given unitage of antitoxin, resulted in death of at least 80% of the mice. The L_o and L_+ toxin doses for a given unitage of standard antitoxin were used as the standard reference for determining the antitoxin titers of the experimental lambs.

The lamb serum samples were titrated for type D antitoxin by comparing their neutralizing power of an L_+ dose of toxin with that of the designated unitage of the standard antitoxin. In each series of titrations of unknown serum, the L_o and L_+ toxin doses for the given unitage of standard antitoxin were included as a control.

RESULTS

Results of the titration of serum samples from 76 normal, nonvaccinated lambs for type D antitoxin are presented (Table 1). Serum samples of 76 lambs were titrated for 0.1 unit of antitoxin per milliliter, and 25 (32.8%) were positive at this level. Serum samples of 21 (27.6%) of the 76 lambs contained 0.25 unit or more of type D antitoxin per milliliter. One-half unit of antitoxin per milliliter of serum was present in 9 (19.1%) of 47 lambs tested. Four of 22 lambs which had negative titers at 0.25 unit of antitoxin per milliliter had positive titers at 0.1 unit.

DISCUSSION

The observation of detectable antitoxin titers in serum samples of 32.8% of the 76 nonvaccinated lambs is believed to have considerable importance, for it stresses the necessity of antitoxin determination of all lambs to be used in enterotoxemia studies. The presence of active antitoxin titers in otherwise normal lambs offers a possible explanation for some of the negative results experienced in earlier attempts to reproduce the disease by intravenous administration of filtrates of intestinal contents of sheep with acute type D enterotoxemia. The development of active antitoxin titers in nonvaccinated lambs would suggest that the animals had previously been exposed to clinical or subclinical enterotoxemia. The results obtained in this study indicate that subacute, clinically inapparent, enterotoxemia may develop in sheep on maintenance rations or on range pastures, and that the feeding of concentrated rations is not essential for the production of type D toxin in the intestinal tract. Worthy of further study would be the incidence of subacute enterotoxemia in lambs being fattened on concentrated rations under feedlot conditions.

SUMMARY

It was found that 32.8% of 76 nonvaccinated lambs had *Clostridium perfringens* type D antitoxin titers of 0.1 unit per milliliter or higher. It is believed that the antitoxin titers resulted from previous contact with the antigenic type D toxin, probably in the form of subacute enterotoxemia.

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SUMMARIO IN INTERLINGUA

Enterotoxemia de Oves. III. Titros de Antitoxina Anti *Clostridium perfringens* Typo D in Normal Non-Vaccinate Agnos

Esseva constatate in un gruppo de 76 non-vaccinate agnos que 32,8% habeva titros de antitoxina anti *Clostridium perfringens* typo D de 0,1 unitates per millilitro o plus. Es opinate que le titros de antitoxina resultava ab previe contactos con le antigenic toxina typo D, probabilemente in le forma de enterotoxemia subacute.

The Isolation of *Vibrio bubulus* (Florent) from Sheep

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DURING A STUDY of the epizootiology of ovine vibriosis, cultures were made (1) from the preputial mucus and semen of rams in both naturally infected flocks and flocks without a history of abortion, and (2) from the vaginas of both aborting and nonaborting ewes. Although *Vibrio fetus* was not isolated from any of the ram mucus or semen cultured, a number of cultures of organisms which resembled *V. fetus* morphologically were isolated. Similar organisms were isolated from vaginal swabs made from aborting as well as nonaborting ewes. It seemed advisable to compare these cultures with strains of Vibrio isolated from other sources. This is a report of the results of cultures made from sheep and the comparative physiology and serology of the organisms isolated.

REVIEW OF LITERATURE

Vibrios other than *V. fetus* have been isolated from the reproductive organs of human beings and cattle. Curtis,[9] in 1913, reported the isolation of a strictly anaerobic Vibrio from leukorrheal discharges of women. This organism was later named *Vibrio mulieris* by Prevot[30] and classed with the nongasogenic anaerobic vibrios. In 1954, Moore[27] described the isolation of vibrios from the vaginas of women attending a fertility clinic. These cultures required a high atmospheric carbon dioxide tension as well as anaerobiosis for primary isolation, failed to reduce nitrate, and did not produce hydrogen sulfide. King[16] reported the isolation of *V. fetus* and "related-vibrio" strains from the blood of human beings. The isolation of *V. fetus* from man had previously been reported.[27, 28]

In 1953, Florent[14] described a Vibrio, isolated from the semen of bulls and the vaginas of cows in Belgium, which was more anaerobic than *V. fetus* and produced an abundance of hydrogen sulfide. A more detailed study of this organism was conducted later by Thouvenot and Florent.[28] They felt that the organism was related to *V. mulieris* (Curtis) Prevot, but that the differences between the 2 organisms were sufficient to justify a new species, and proposed the name *Vibrio bulbulus*.

Bryner and Frank[7] studied Vibrio cultures from aborted bovine fetuses and the reproductive organs of bulls and cows. All of the vibrios isolated from aborted fetuses were catalase-positive, hydrogen sulfide-negative, failed to grow in deep stab cultures, and were considered to be *V. fetus*. Vibrios from the reproductive organs which met these qualifications were also considered to be *V. fetus*. Vibrios which produced hydrogen sulfide but not catalase and showed light growth in deep stabs were not considered to be *V. fetus*.

Price *et al.*,[31] in a similar study of bovine cultures, found strains of *V. fetus* of fetal origin and unidentified Vibrio cultures which were serologically related to *V. fetus* strains known to be hydrogen sulfide-negative. Three strains which were not serologically related to *V. fetus* were hydrogen sulfide-positive and, as none of these strains were isolated at abortion, they were not considered to be *V. fetus*.

In Norway, Lindqvist[22] isolated 7 strains of Vibrio, catalase-negative and hydrogen sulfide-positive, from the semen and cervical mucus of cattle. Antigens prepared from these strains were not agglutinated by positive vaginal mucus from cows infected with *V. fetus*. He considered the cultures to be nonpathogenic.

In 1956 and 1957, a number of other authors reported the isolation of Vibrio other than *V. fetus* from the genital organs of cattle.[1, 5, 17, 26, 29, 32, 33]

Kuzdas and Morse[21] reported a study made on 66 cultures of Vibrio isolated from animals and man, as well as from soil, water, and cheese. All cultures were catalase-positive, with the exception of 1 strain of human origin and 8 strains isolated from bovine vaginal mucus or semen. The 8 strains of animal origin produced hydrogen sulfide and tolerated mediums with a sodium chloride concentration of 3.5%, while known *V. fetus* strains would not grow in the presence of more than 2.5% sodium chloride.

In 1959, DiLiello *et al.*[10] reported biochemical and serologic studies on 68 cultures of Vibrio from various sources. An unspecified number of cultures from nonfetal bovine, ovine, and human sources were found to be catalase-negative, hydro-

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gen sulfide-positive, sodium chloride tolerant, and serologically unrelated to known *V. fetus* strains.

Binns and Thorne[3] isolated vibrios from the preputial mucus of rams in 1954. Later in the same year, similar organisms were isolated at the Montana Veterinary Research Laboratory.[12] In both instances, the organisms were found to be hydrogen sulfide-positive and catalase-negative.

In 1957, Eide and Helle[11] described an epizootic of ovine vibriosis in Norway due to a Vibrio which did not produce hydrogen sulfide, produced only small amounts of catalase, and did not grow in deep stabs. Vibrios were isolated from aborted lambs, vaginal mucus of ewes which produced healthy lambs, vaginal mucus of virgin ewes, fetal fluid from normally lambing ewes, gastric contents of normal lambs at birth, and the semen of rams. Cross-agglutination tests indicated serologic relationships between the isolates.

More recently, these authors reported an investigation of the same flock during a 1½-year period following the abortion epizootic.[12] They were able to isolate vibrios from vaginal mucus and from material taken at normal parturition, although there were no clinical signs of vibriosis during this time. The vibrios were considered to be similar to the strain isolated at the time of the abortions, but were even less active in the production of catalase, had light growth in deep stabs, and had lower pathogenicity for pregnant guinea pigs.

In 1960, Vibrio organisms which produced both hydrogen sulfide and catalase, and were inhibited by 2.0% sodium chloride, were isolated from ovine fetuses aborted in 2 flocks in Kentucky.[6]

MATERIALS AND METHODS

Isolation Technique.—Preputial mucus was collected from rams, using methods and instruments described by Binns and Fincher[4] for collecting samples from bulls. After the mucus was collected, the instrument was rinsed with 1 ml. of nutrient broth which was then distributed in 4 to 7 tubes of semisolid medium made from Brucella broth* to which 0.15% agar and 10% normal horse serum had been added. The atmosphere under which the semisolid cultures were incubated did not appear to be important, but in most instances incubation was carried out in partially evacuated anaerobe jars. After 4 or 5 days of incubation, smears were made, and cultures which contained vibrios or were considered suspicious were subcultured in the following selective medium:

Albimi Brucella broth as base
Agar, 0.15%
Ethyl violet, 1:800,000
Brilliant green, 1:100,000
Bacitracin, 20 units per ml.
Actidione, 0.1 mg./ml.

This medium, which was inspired by the selective Vibrio medium of Kuzdas and Morse,[21] proved to be of considerable value in reducing the number of contaminants prior to plating. For a short time at the beginning of the experiment a very similar medium, which was a modification of the Kuzdas and Morse[20] Brucella medium, was used. More recently, 100 to 200 mg. of ristocetin* per milliliter of medium has been substituted for ethyl violet with good results. Because Vibrio organisms are partially inhibited by the medium, duplicate cultures in plain semisolid medium also were made. After an incubation period of 3 or 4 days, tubes having satisfactory Vibrio growth as compared to contaminant growth were streaked on Difco blood agar** plates containing 5% defibrinated bovine blood. The plates were incubated for 5 or 6 days under an atmosphere reduced with a vacuum pump to a pressure of 275 mm. of mercury. It was sometimes necessary to make several plating trials before colonies were obtained.

Semen was collected by electronic ejaculation and was cultured using the methods employed for preputial mucus.

Vaginal and uterine cultures were made with cotton-tipped wire swabs inserted through a glass speculum. The swabs were washed off with broth which was then cultured on plates and in semisolid medium.

Cultures from aborted fetuses were made in semisolid medium and on blood agar plates incubated under 15% carbon dioxide tension.

Preparation of Antigens and Antiserums.—Growth for antigen was produced in 1,500-ml. Erlenmeyer flasks containing 200 ml. of Brucella broth (Albimi). The 48-hour growth from 4 slants was used as inoculum, and the atmosphere in the flask was reduced to a pressure of 275 mm. of mercury. The flask was incubated at 37 C. on a shaker for 24 or 48 hours. The growth was killed with 0.3% formalin and washed twice in formol-saline solution of the same concentration. The final density of the suspension was adjusted photoelectrically to a value between McFarland tubes 1 and 2.

Antiserums were produced by subjecting rabbits to a series of immunizing doses of formalin-killed organisms. Agglutination trials were set up in serial dilution with essential controls. Readings were made after 40 to 48 hours of incubation at 37 C.

Biochemical Reactions and Cultural Characteristics.—Difco nutrient gelatin with 10% horse serum added was used to check for gelatinase production. The tubes were examined after 21 days' incubation.

Because dehydrated litmus milk was found to give inconsistent results, 0.1% azolitmus and 0.1% agar were added to skim milk, which was then sterilized. This medium was found to be satisfactory, and final observations were made on the cultures after 21 days' incubation.

* Albimi Laboratories, Brooklyn, N.Y.

* Spontin, Abbott Laboratories, North Chicago, Ill.
** Difco Laboratories, Detroit, Mich.

Indole-nitrate broth * was used for the indole production and nitrate reduction tests. After 5 days' incubation, duplicate tubes were checked for indole by the Bohme test and for nitrite by the Wallace and Neave test.[20] A number of cultures were also checked for indole with the Gnezda paper strip test.[20]

Hydrogen sulfide production was detected by the incubation of cultures in tubes of semisolid medium with strips of lead acetate-impregnated filter paper suspended over the medium. Examination was made after 48 hours' incubation.

Phenol red broth base ** with 1.0% trypticase and 0.5% yeast extract was used as a basal medium for fermentation studies. After sterilization, the following filter-sterilized saccharides and alcohols were added in amounts sufficient to give a final concentration of 0.5%: xylose, rhamnose, glucose, sucrose, lactose, glycerol, sorbitol, and mannitol. Incubation was carried out in air without added carbon dioxide. Examination of the tubes was made after 7 and 14 days of incubation. At the final examination, a Beckman Model G pH meter was used to check tubes having slight color change. Controls consisted of uninoculated tubes for each carbohydrate and an inoculated tube of broth base without carbohydrate for each strain used.

Catalase production was detected by mixing on a slide a small amount of 72-hour growth from a slant with 3% hydrogen peroxide. Invariably, the catalase-positive strains were strongly positive.

Blood agar base (Difco), to which was added 0.2% yeast extract, 0.2% soluble starch, and 5.0% defibrinated bovine or rabbit blood, was used to determine the effects of the organisms on blood agar. Plates were examined after 5 to 7 days' incubation.

Sodium chloride tolerance was determined by a method similar to that used by Kuzdas and Morse.[21]

The atmospheric requirements were determined in deep stab thiole semisolid cultures as described by Bryner and Frank,[7] and by the use of blood agar plates incubated in air, under 15% Co_2 tension, and under anaerobic conditions. Some of the strains were also checked under anaerobic conditions with added CO_2.

Flagella were stained by the method of Casares-Gil.[20] Cell measurements were made with a filar micrometer. The organisms were stained with the cell wall method of Knaysi,[19] and the average measurement of 10 different cells was calculated.

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RESULTS

CULTURES FROM RAMS

Prepuce Cultures.—During the period from the spring of 1954 through the spring of 1959, cultures were made from the preputial mucus of 85 rams on 12 different ranches which were having losses due to vibriosis (Table 1). An attempt was made each spring to select for investigation ranches which had histories of vibriosis following the introduction of new rams, and which still had all or most of the rams available for culture.

Vibrio organisms were isolated from 40 of the rams. All isolates produced hydrogen sulfide, did not produce catalase, and had light growth in deep stabs. On the basis of examinations of stained smears, cultures from 18 rams were considered suspicious for Vibrio organisms, but isolations could not be made due to heavy contamination. Cultures from 27 of the 85 rams were negative for vibrios.

During the period of this study, cultures were made from the preputial mucus of rams obtained each fall from the Animal Industry Department at Montana State College to breed ewes owned by this laboratory. Vibrios were obtained from 14 of 49 rams cultured (Table 1). The morphologic and physiologic characteristics of

TABLE 1—Isolation of Vibrio from the Preputial Mucus and Semen of Rams

	Preputial cultures				Semen cultures			
	Ranches		A. I. Department*		Ranches		A. I. Department*	
Finding	No. rams	%	No. rams	%	No. rams	%	No. rams	%
Rams negative	27	31.8	25	51.0	10	30.3	17	63.0
Rams suspicious**	18	21.2	10	20.4	5	15.2	5	18.5
Vibrio isolated	40	47.0	14	28.6	18	54.5	5	18.5
Total	85	100.0	49	100.0	33	100.0	27	100.0

* Rams obtained from Animal Industry Department, Montana State College.

** Cultures were considered suspicious for Vibrio, but due to heavy contamination isolation could not be made.

these cultures were identical with those of the cultures isolated from rams on ranches with vibriosis. The last known abortion due to vibriosis in the ewes maintained by the Animal Industry Department occurred in 1953, 1 year before the ram culture study was started.

Semen Cultures.—In the fall of 1957, semen as well as preputial mucus cultures were made from rams obtained from the Animal Industry Department. In the spring of 1958, and again in 1959, similar studies were conducted on 33 rams from 8 ranches with histories of vibriosis (Table 1). Vibrios were isolated from the semen of 5 of the 27 rams from the Animal Industry Department and from 18 of the 33 rams from ranches. In most instances where isolations were made from semen, preputial cultures were also positive. All of the cultures isolated were hydrogen sulfide-positive and catalase-negative.

CULTURES FROM EWES AND LAMBS

Isolations During an Epizootic.—During an epizootic of vibriosis in the spring of 1955, 20 ewes which had not yet aborted or lambed were ear-tagged and placed under close observation. Thirteen of the ewes gave birth to normal lambs, 6 to weak lambs, and 1 to a stillborn lamb. Two of the weak lambs were killed, and 4 died shortly after birth. Cultures were made from these lambs and from the stillborn lamb (Table 2). *Vibrio fetus* was isolated from 3 of the weak lambs, while cultures from the other 3 were negative. A Vibrio was isolated from the stillborn lamb but

TABLE 2—Lambing and Culture Record During and Following a Vibriosis Epizootic

	1955										1956	
Ewe No.	Lamb condition at birth	Lamb culture	Vaginal cultures*								Lamb condition at birth	Vaginal culture*
101	Weak–died	—	1 Vf	8 Vf	36 —	46 —	143 —	....	....	.....	Normal	1 Vb
102	Normal	....	1 —	28 —	....	....	....	....	....	.....	Normal	..
103	Weak–died	—	1 —	33 —	....	....	....	....	....	.....	.....	..
104	Normal	....	1 —	41 —	....	....	....	....	....	.....	Dry	..
105	Normal	....	2 —	49 —	....	....	....	....	....	.....	Normal	..
106	Normal	....	1 Vf	18 —	48 —	153 —	....	....	....	.....	Normal	1 —
107	Normal	....	1 —	38 —	....	....	....	....	....	.....	Normal	..
108	Normal	....	1 Vf	15** +	30 Vb	43 —	53 —	128 —	....	.....	Normal	6 Vb
109	Normal	....	2 —	47 —	....	....	....	....	....	.....	Normal	..
110	Normal	....	1 Vb	6 Vb	17 Vb	32 —	42 Vb	56 —	77 —	139 —	Normal	3 —
112	Normal	....	1 —	38 —	....	....	....	....	....	.....	Normal	..
113	Normal	....	2 Vf	19 Vb	34 Vb	47 Vb	57 Vb	71 —	92 —	154 Vb	Normal	1 —
114	Stillborn	+†	1 Vf	11 Vb	26 —	41 —	....	....	....	.....	Normal	..
115	Weak–killed	Vf	1 Vf	7 Vf	16 —	44 —	....	....	....	.....	Normal	..
116	Normal	....	3 Vb	20 Vb	35 —	48 Vb	56 Vb	72 Vb	93 —	155 —	Ewe died‡	..
117	Weak–killed	—	39 —	52 —	....	....	....	....	....	.....	.....	..
118	Weak–died	Vf	1 Vf	11 Vf	20 Vb	33 —	48 —	....	....	.....	Dry	..
119	Normal	....	1 Vf	10 Vb	25 Vb	38 Vb	48 Vb	62 Vb	83 —	145 Vb	Normal	1 —
120	Normal	....	1 —	38 —	....	....	....	....	....	.....	Normal	..
121	Weak–died	Vf	1 Vf	7 Vf	15 —	44 —	....	....	....	.....	Dry	..

Vf = *Vibrio fetus*; Vb = *Vibrio bubulus*.

* Numbers indicate the number of days after lambing that swab cultures were made; finding is indicated below the day.

** H_2S +, catalase +, Vibrio (6468).

† Cultures from the fetus were positive for Vibrio, but were lost before identification could be made.

‡ Ewe 116 died on 4–8–56, possibly due to bloat. Cultures from twin fetuses in the uterus were negative for Vibrio.

was lost before it could be identified. It is probable that this organism was *V. fetus*, inasmuch as a vaginal swab from the ewe was positive.

Vaginal swabs were made from the ewes shortly after lambing and at intervals during the summer. In most instances, vaginal swabbing of individual ewes was discontinued after 2 or 3 successive negative cultures were obtained. *Vibrio fetus* was isolated (Table 2) shortly after lambing from the ewe which had the stillborn lamb (114), from 4 ewes which gave birth to weak lambs (101, 115, 118, and 121), and from 4 ewes with normal lambs (106, 108, 113, and 119).

Vibrios which produced hydrogen sulfide but did not produce catalase were isolated from the vaginas of 2 ewes (110 and 116) shortly after normal lambing (Table 2). Similar organisms were isolated, as a result of follow-up cultures, from 5 ewes (108, 113, 114, 118, and 119) which had been positive for *V. fetus* when cultures were made shortly after lambing.

One ewe (108) which gave birth to a normal lamb was positive for *V. fetus* 1 day after lambing. However, a Vibrio which produced both hydrogen sulfide and catalase was isolated from a vaginal swab made 15 days later, and a 3rd Vibrio which was hydrogen sulfide-positive and catalase-negative was isolated 30 days after lambing.

Seventeen of the 20 ewes were kept through the next year (Table 2). Fourteen of these ewes lambed normally, and 3 were dry. Vaginal swabs were made only from the ewes which had been found infected with one or the other of the Vibrio species the previous year. Vibrios which produced hydrogen sulfide and did not produce catalase were isolated from 2 ewes (101 and 108).

Isolations Following Exposure to Contaminated Pasture.—In the fall of 1955, 52 virgin yearling ewes were bred with 2 rams which had been found free of preputial infection. Following breeding, 25 of the ewes were placed in a small, poorly drained pasture where a flock of 500 ewes had been held the previous spring (1955), during an epizootic of vibriosis. The water supply was a small, slowly running stream. It was felt that holding the ewes from early winter through lambing time on ground which had been heavily contaminated would provide information on the significance of premises infection. The remaining 27 ewes were held at the laboratory as a control group.

As the ewes lambed, they were returned to the laboratory where vaginal swabs were made for culture. Twenty-one ewes lambed normally; 1 aborted a dead lamb in late pregnancy; and 3 gave birth to full-term, living lambs which died shortly after birth, presumably due to exposure to cold weather. Cultures from the aborted lamb were negative for Vibrio, as were cultures from 2 of the full-term lambs. Cultures from the abomasum of the other full-term lamb yielded a Vibrio (isolate 6912, ewe B-7019) which produced both hydrogen sulfide and catalase. Vibrios which produced hydrogen sulfide but did not produce catalase were isolated from the vagina of ewe B-7019 and from the vaginas of 2 other ewes, both of which gave birth to normal lambs. A Vibrio (6943) isolated from another normally lambing ewe produced hydrogen sulfide as well as catalase.

Twenty-six of the control ewes lambed normally, and 1 ewe gave birth to a dead full-term lamb after a difficult delivery. Cultures from the lamb were negative. Vaginal cultures were made from 22 of the ewes following lambing, and vibrios were isolated from 4. Three isolates were hydrogen sulfide-positive and catalase-negative, and 1 (6986) produced both hydrogen sulfide and catalase. All 4 grew in deep stabs and were tolerant of 3.5% sodium chloride.

PHYSIOLOGY

Vibrio fetus of Ovine Origin.—Fifteen cultures of *V. fetus* isolated from aborted ovine fetuses were found to produce catalase, reduce nitrate to nitrite, reduce litmus milk, and grow on the surface or just below the surface of deep stabs (Table 3). Indole and gelatinase were not produced. Three strains produced small amounts of hydrogen sulfide, while 12 were negative. All of the cultures were very sensitive to sodium chloride, having only traces of growth at the 1.5% level. Of 10 cultures which were streaked on bovine blood plates, 7 did not cause any change in the blood, 2 caused weak *beta* hemolysis, and 1 produced very slight green discoloration. Of 7 cultures streaked on rabbit blood, 6 did not cause change and 1 caused weak *beta* hemolysis. Growth on solid agar was good

under either 15% carbon dioxide tension or an atmosphere reduced to a pressure of 275 mm. of mercury. Most cultures grew in tubes of semisolid medium in air, but growth was enhanced by reduction of the oxygen tension. Some of the cultures had light growth on slants incubated in air, while others did not grow.

Vibrio fetus of Bovine Origin.—Ten cultures of *V. fetus* isolated from bovine fetuses were found to be similar to *V. fetus* of ovine origin in the physiologic tests to which they were subjected, except that none produced hydrogen sulfide (Table 3). The diately after they were received, but good growth was obtained under an atmosphere reduced to a pressure of 275 mm. of mercury. After they had been carried on mediums for some time, growth on plates was obtained under 15% carbon dioxide tension. Growth under strictly anaerobic conditions was very poor.

Vibrios Isolated from Bulls.—Four of 5 Vibrio cultures isolated from bulls in the United States gave physiologic reactions similar to those of the known *V. bulbulus* cultures obtained from France (Table 3). The 5th culture (5712) pro-

TABLE 3—Physiologic Reactions of Vibrios from Various Sources

Vibrio type	Source	No. of strains	H_2S	Catalase	Deep stab	Sodium chloride tolerance				
						0	1.5%	2.5%	3.5%	4.5%
V. fetus	Ovine fetus	15	−(12) ±(3)	+	Surface	+	±	−	−	−
V. fetus	Bovine fetus	10	−	+	Surface	+*	±	−	−	−
V. bubulus	Bovine genitals, France	4	+	−	Surface and stab	+	+	+	±	−
V. bubulus	Bovine genitals, U.S.A.	5	+	−	Surface and stab	+**	+	+	±	−
V. bubulus	Ram prepuce, Montana	29	+	−	Surface and stab	+	+	+	±	±
V. bubulus	Ram semen, Montana	34	+	−	Surface and stab	+	+	+	±	±
V. bubulus	Vaginas of ewes, Montana	18	+	−(13) +(5)	Surface and stab	+	+	+	+	±
?	Ovine fetus (185-54), Norway	1	+	−	Surface and stab	+	+	+	±	−
?	Ram semen, (Modell), Norway	1	+	−	Surface and stab	+	+	+	±	−

All strains reduced nitrate to nitrite, did not produce indol, and reduced litmus.
* Only 6 strains checked for sodium chloride tolerance.
** Strain 5712 was inhibited at the 1.5% level.

6 cultures which were checked for sodium chloride tolerance had little or no growth at the 1.5% level. Upon initial isolation, most of the cultures seemed to be more fastidious than ovine isolates.

Vibrio bulbulus Cultures.—Four cultures of *V. bulbulus* (Mix, Waterloo, Atome, Imperial) which were obtained from the Pasteur Institute produced hydrogen sulfide and did not produce catalase. All 4 reduced nitrate to nitrite, reduced litmus, and did not produce indole. Two cultures grew in the presence of 3.5% sodium chloride, and 2 grew at the 2.5% level but not at the 3.5% level (Table 3).

All 4 cultures had surface and light stab growth in deep thiole stabs incubated under 10% carbon dioxide tension. None of the cultures grew on plates either in air or under 15% carbon dioxide tension imme-

duced hydrogen sulfide, did not produce catalase, grew in deep stabs, but was inhibited by 1.5% sodium chloride. This organism grew poorly on all the mediums employed for conducting physiologic tests, so the lack of growth in the presence of sodium chloride may not be of special significance. Its nutritional requirements were apparently somewhat different from those of the other organisms tested in this group.

Vibrios Isolated from Rams.—Twenty-nine Vibrio cultures isolated from the preputial mucus of rams gave physiologic reactions identical to those of *V. bubulus* (Table 3). All had some growth in the presence of 3.5% sodium chloride, and a few had light growth at the 4.5% level. On primary isolation, little or no growth was obtained on solid mediums under 15% carbon dioxide tension, but excellent growth was obtained in jars evacuated to

a pressure of either 275 or 140 mm. of mercury. Growth under strictly anaerobic conditions (jars evacuated, flushed with hydrogen, and evacuated again) was quite poor. Ten strains grown in mediums containing carbohydrates did not produce evidence of fermentation.

Thirty-four cultures isolated from the semen of rams were found to have physiologic characteristics identical to those of the cultures isolated from preputial mucus (Table 3).

Vibrio Isolated from Ewes and Lambs.—Eighteen cultures of Vibrio isolated from the vaginas of ewes and found to be hydrogen sulfide-positive were subjected to additional study. All reduced nitrates to nitrites, reduced litmus, grew in deep stabs, did not produce indole, and grew in the presence of 3.5% sodium chloride (Table 3). Thirteen strains checked in mediums containing carbohydrates had no evidence of fermentation.

Five cultures (6005, 6468, 6943, 6961, and 6986) produced catalase. One of the catalase-producing cultures (6005) was recovered from the vagina of an ewe following the birth of a normal lamb in the spring of 1953. The ewe had aborted due to vibriosis in 1952. Two cultures (6468 and 6961) were recovered from ewes which gave birth to normal lambs in a flock which was suffering from an epizootic of vibrionic abortion at the time. One of these ewes (6468) was positive for *V. fetus* when a vaginal culture was made 14 days earlier, shortly after lambing. Two cultures (6943 and 6986) were isolated following the birth of normal lambs from ewes on a pasture which had been heavily contaminated with *V. fetus* during a natural epizootic of vibriosis the previous spring. One of these cultures (6943) produced hydrogen sulfide in only small amounts but, due to its ability to grow in deep stabs and in the presence of 3.5% sodium chloride, it was not considered to be *V. fetus*.

A Vibrio (6912) which produced both hydrogen sulfide and catalase was isolated from the abomasum of a full-term lamb which died shortly after birth, probably due to exposure. This isolate grew in deep stabs and was tolerant of 3.5% sodium chloride.

Norwegian Vibrios.—In June of 1959, lyophilized Vibrio cultures 185-54 and "Modell" were received from the Norges Veterinaerhøgskole, Institut for Sekualfysiology, Oslo, Norway. Strain 185-54 had been isolated from an ovine fetus and Modell from ram semen during the investigation of an epizootic of vibriosis at an experiment station in Norway. Both of these strains, as well as numerous other cultures isolated during the investigation, were reported to be hydrogen sulfide-negative, very weak or negative for catalase production, unable to grow in deep stabs, and serologically related.[11]

With the methods used at our laboratory, we found both strain 185-54 and Modell positive for hydrogen sulfide, negative for catalase, able to maintain light growth in deep stabs, and tolerant of sodium chloride at the 2.5% level (Table 3). It would appear that these organisms should be considered to be *V. bubulus*.

MORPHOLOGY

Smears of vibrios which produced hydrogen sulfide, grew in deep stabs, and tolerated more than 2.5% sodium chloride revealed comma, "sea-gull," and dark-staining coccoid forms similar to those seen in *V. fetus* cultures (Fig. 1). Occasionally, longer forms were seen which consisted of several wave lengths and resembled the "filamentous" forms sometimes seen in cultures of *V. fetus*. The comma forms were frequently quite short and usually appeared somewhat straighter than *V. fetus* comma forms. The comma forms were 2.5 to 3.3 μ in length and the longer forms were 4.7 to 8.6 μ. Comparable measurements were obtained from *V. fetus* cultures. As the cultures grew older, organisms with dark-staining terminal granules were sometimes seen, and free coccoid forms were more numerous (Fig. 2).

Dark-staining coccoid forms were frequently observed in *V. fetus* cultures and appeared to be more common in cultures belonging to serotype I than in cultures of other serotypes (Fig. 3, 4). After several days' incubation, larger, paler-staining, round, "ghost" forms often constituted the majority of the organisms present (Fig. 5). In some cultures, forms which resembled a ring with a pale center and a dark-staining body at one side were sometimes found (Fig. 6).

Flagella stains of *V. fetus*, *V. bubulus*, and related vibrios showed that comma forms have a single polar flagellum, while

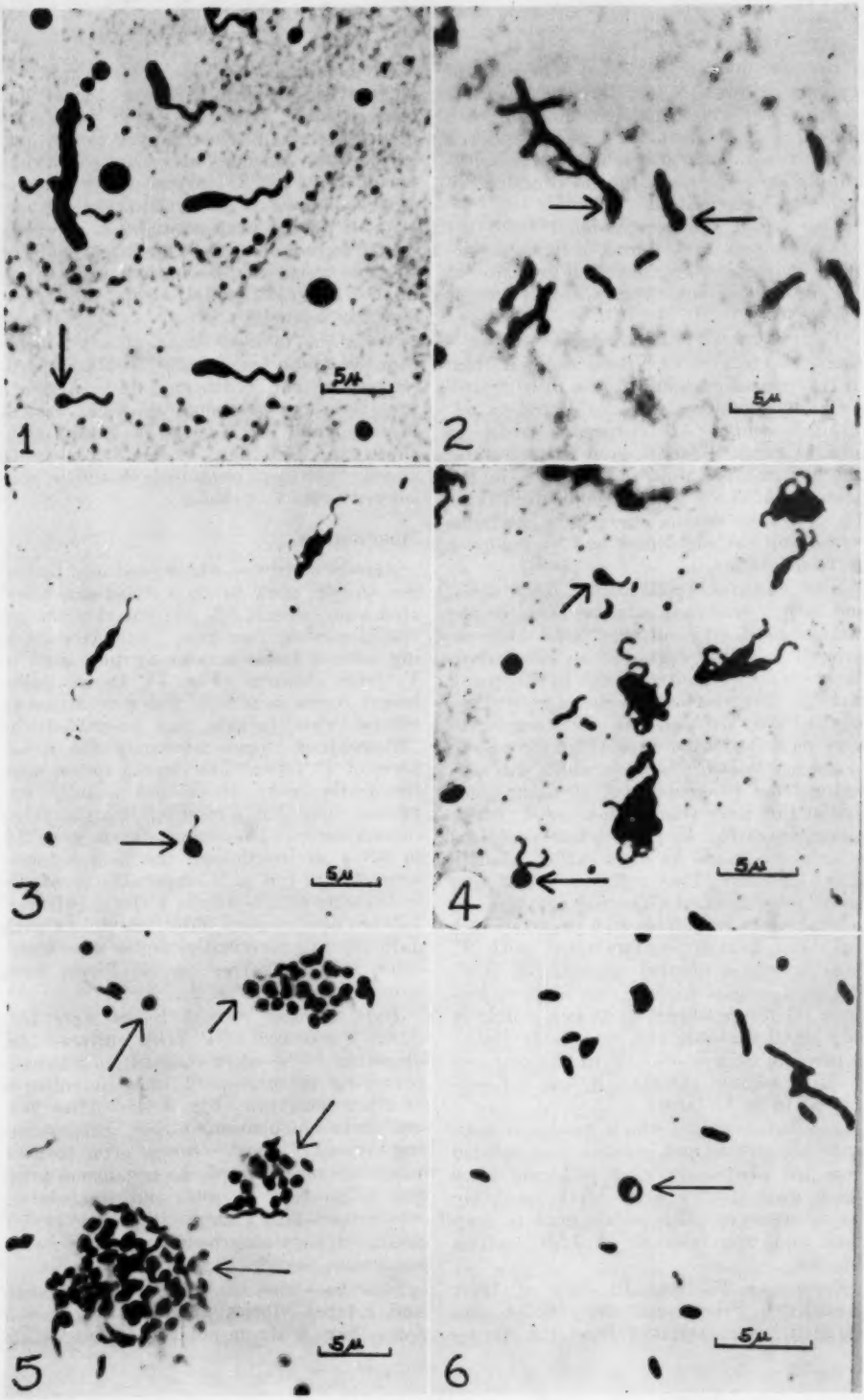
1
5μ
2
5μ
3
5μ
4
5μ
5
5μ
6
5μ

TABLE 4—Cross-Agglutination Reactions of Vibrio fetus

Antigen	Origin	Sero-type	Serums 4849	5854	4777	2440	4440	2221	5049	5090	6333	7360	2315	5380	4404	5648	5367
4849	Ovine fetus	I	100	0	100	400	0	0	0	0	0	0	0	0	0	0	0
5854	Ovine fetus	I	800	400	100	400	0	0	0	0	0	0	0	0	0	0	0
4777	Ovine fetus	I	800	50	800	100	800	50	0	0	0	0	0	800	800	0	0
2440	Ovine fetus	I	400	50	0	200	0	0	0	0	0	0	0	0	0	0	0
4440	Ovine fetus	II	0	0	0	0	400	200	50	0	0	0	0	50	400	800	0
2221	Ovine fetus	II	0	0	0	0	800	1,600	0	0	0	400	200	0	1,600	400	0
5049	Ovine fetus	V	0	0	0	0	0	0	400	0	400	25	0	0	0	0	0
5090	Ovine fetus	V	0	0	0	0	0	100	800	1,600	3,200	25	0	0	0	200	0
6333	Ovine fetus	V	0	0	0	0	0	0	1,600	200	3,200	200	0	0	0	1,600	200
7360	Ovine fetus	V	0	0	0	0	200	0	200	400	0	200	0	0	0	0	0
2315	Bovine fetus	III	0	0	0	0	100	0	800	3,200	200	200	400	0	0	1,600	1,600
5380	Bovine fetus	III	0	0	0	0	800	0	0	0	0	0	25	800	0	200	200
4404	Bovine fetus	III	0	0	0	0	0	0	0	0	0	0	0	100	400	100	0
5648	Bovine fetus	III	0	0	0	0	0	0	0	0	0	0	0	0	0	200	0
5367	Bovine fetus	III	0	0	0	0	0	0	0	0	0	0	0	0	100	400	400

Figures indicate the reciprocal of the highest dilution in which complete agglutination occurred.

"sea gull" and longer forms usually have one at each pole. The dark coccoid bodies frequently possessed a flagellum, and motility could be observed in hanging drop preparations (Fig. 1, 3, 4). The coccoid stage of vibrios has been investigated by Hallock.[16] Electron photomicrographs prepared by Rhoades[33] revealed coccoid forms bearing flagella.

Serology

A cross-agglutination study was made of 33 representative Vibrio strains, using antigens and rabbit antiserums prepared with formalin-killed whole cells. If the vibrios which produce hydrogen sulfide and are tolerant of 2.5% sodium chloride are considered as one group, the cross-agglutination reactions within the group were not numerous, indicating a heterogeneity of serotypes. In strains in this group there was only slight relationship to *V. fetus* strains of either ovine or bovine origin, but ovine *V. fetus* strain 4777 was agglutinated by semen serum from the sodium chloride-tolerant vibrios.

The French strains of *V. bubulus* were related to a number of the vibrios of ram and bull origin isolated in the United States, and to Norwegian strains 185-54 and Modell.

The *V. fetus* isolates of ovine origin could be assigned to 2 groups which had little or no serologic relationship to each other, and a smaller group which had some relationship to both of the other groups (Table 4). According to the classification of Marsh and Firehammer,[24] the 2 main groups would be types I (4849, 5854, 4777, and 2440) and V (5049, 5090, 6333, and 7360); the smaller group would correspond to type II (4440 and 2221). There was some cross agglutination between the *V. fetus* strains of ovine and bovine (type III) origin.

Discussion

Vibrios which produce hydrogen sulfide, do not produce catalase, are tolerant of 2.5% or more sodium chloride, and show light growth in deep stab cultures probably should be tentatively identified as *Vibrio bubulus* Florent.[35] The frequency

Legends for illustrations (left-hand page)

Fig. 1—Vibrio bubulus isolated from the prepuce of a ram. Casaeres-Gil stain. Notice flagellated coccoid form. Large round bodies in this preparation are artifacts.

Fig. 2—Vibrio bubulus isolated from the prepure of a ram. Carbol fuchsin stain. Notice terminal granules.

Fig. 3 and 4—Vibrio fetus, ovine origin. Casaeres-Gil stain. Notice flagellated coccoid forms.

Fig. 5—Vibrio fetus, ovine origin, 3-day slant growth. Carbol fuchsin stain. Dark coccoid and pale ghost forms.

Fig. 6—Vibrio fetus, ovine origin, 24-hour slant. Notice ring form.

with which such organisms are isolated from animal sources makes it imperative that they be given a name rather than repeatedly resorting to a cumbersome description of physiologic characteristics in order to describe the isolates. If this name does not withstand the test of time, it could be dropped and the organisms previously so identified could be reclassified. At the present time, the differences between *V. bubulus* and *V. fetus* appear to be sufficient to justify the existence of separate species. If evidence should be presented demonstrating transformation or transduction from one type to the other, this viewpoint would have to be modified.

The isolation of vibrios which resemble *V. bubulus* except for the production of catalase poses an additional problem. Inasmuch as they resemble *V. bubulus* more closely than *V. fetus*, they probably should be placed in the former group. The possibility that such cultures may actually consist of a mixture of both types of vibrios should be kept in mind, and extensive trials should be made with picked colonies before identification. Due to the production of small amounts of hydrogen sulfide by some strains of *V. fetus*, additional tests such as sodium chloride tolerance and growth in deep stabs should be used. Great care must be used in the interpretation of the results of deep stab trials, since the growth is usually very light and may be confused with inoculum left by the loop or needle.

As a result of the isolation of *V. bubulus* from rams, ewes, and lambs, it would appear that this organism is ubiquitous in sheep. The percentage of isolations from rams associated with vibriosis epizootics was considerably higher than that of isolations from rams obtained from the supposedly clean flock of the Animal Industry Department (Table 1). This may not be of special significance, inasmuch as the rams from ranches with histories of vibriosis represented many sources, while the rams from the flock of the Animal Industry Department represented but one source, a source with management practices possibly somewhat different from those found on most farms and ranches. The failure to isolate *V. fetus* from rams on ranches with histories of vibriosis is evidence that the ram is probably not an important source of ewe infection.

The isolation of *V. bubulus* from the vaginas of 5 ewes which had previously been found infected with *V. fetus* is intriguing and would tempt one to consider the possibility of change in the physiologic properties of vibrios within the animal (Table 2). Such a possibility has been suggested by Bond.[5] However, it is conceivable that some other phenomenon took place, such as overgrowth of *V. bubulus* by *V. fetus* during the time *V. fetus* was present in the vagina after abortion or lambing, followed later by a decrease in the *V. fetus* population and an increase in *V. bubulus*, which may be better suited to survival in the vagina.

Perhaps the most perplexing history is that of ewe 108, which yielded *V. fetus* on vaginal culture 1 day after lambing, a Vibrio which produced both hydrogen sulfide and catalase on the 15th day, and typical *V. bubulus* when cultures were made 30 days after lambing (Table 2).

The isolation of *V. fetus* from the vaginas of 4 ewes which gave birth to apparently normal lambs could indicate low pathogenicity of the strain involved, resistance of the ewe or, possibly, infection too late in pregnancy to cause death of the lamb before it was born (Table 2).

The absence of abortions in 1956 in the 17 ewes held over from the flock which was found to be naturally infected in 1955 is in accord with previous findings of the authors and others (Table 2).[25,39] The incidence of dry ewes was perhaps higher than is usually found in such instances. Vibrionic abortion seldom occurs in successive years in the same flock, and lambing percentages are usually near normal the year following an epizootic.

The isolation of *V. bubulus* from both control ewes and ewes exposed to ground previously contaminated with *V. fetus* is compatible with the isolation of the same organism from rams in flocks with and without histories of abortion. The failure of the ewes in the exposed group to show evidence of vibriosis would appear to indicate that infected ground is not a hazard to pregnant ewes during the season following an epizootic. However, the histories of some field epizootics tend to cast some doubt on this observation. An investigation of *V. fetus* survival times in soil and water would probably yield information of value.

The investigation of many epizootics of ovine vibriosis in Montana over a period

of years has failed to incriminate *V. bubulus* as a cause of abortion. However, in view of recent reports of ovine abortion due to vibrios which are similar to *V. bubulus* in some respects, the possibility of pathogenic potential should not be disregarded.[6,11]

On the basis of cross-agglutination studies using antigens and serums prepared from formalin-killed whole cells, it is apparent that the *V. bubulus* group encompasses a number of serotypes. Frank *et al.*,[15] as well as Biberstein,[2] found 2 serotypes in catalase-negative vibrios from bovine sources. We found very little serologic relationship between *V. bubulus* and *V. fetus* strains of ovine and bovine origin, which is in agreement with the findings of Biberstein.[2] Ristic *et al.*[34] demonstrated related heat-stable antigens in a saprophytic Vibrio and in *V. fetus* of bovine origin.

The work reported here is not extensive enough to elucidate the antigenic pattern of *V. bubulus*, but it does indicate that such organisms have very little relationship with known *V. fetus* serotypes and that there is considerable diversity within the *V. bubulus* group itself.

The *V. fetus* serotype classification of Marsh and Firehammer[24] probably should be modified somewhat, omitting types II and IV and assigning these strains to types I or V. Most bovine strains fall in type III, which is related to type V. Additional work is required to determine whether both type III and type V should be retained as separate types or should be combined as one type and a subtype.

SUMMARY

Cultures were made from the preputial mucus of 85 rams on 12 ranches and from the semen of 33 rams on 8 ranches immediately following epizootics of vibrionic abortion. In no instance was *Vibrio fetus* isolated, but 40 cultures which were hydrogen sulfide-positive and catalase-negative and had light growth in deep stab cultures were isolated from preputial mucus; 18 similar cultures were isolated from semen. All cultures tolerated 2.5% or more of sodium chloride. Similar organisms were isolated from the preputial mucus and semen of rams from a flock without recent history of abortion.

On the basis of physiologic tests, the cultures isolated from rams appeared to be identical to 4 strains of *Vibrio bubulus* (Florent) of bovine origin which were obtained from France. It is suggested that vibrios of ovine origin which are physiologically comparable to *V. bubulus* should be so named. At present, the differences between *V. fetus* and *V. bubulus* appear to be sufficient to justify the existence of separate species. Vibrios which produce catalase but have the other physiologic characteristics of *V. bubulus*, such as tolerance of 2.5% or more of sodium chloride and ability to grow in deep stab cells, more nearly resemble *V. bubulus* than *V. fetus* and should tentatively be placed in the former species. The fact that known *V. fetus* cultures may produce small amounts of hydrogen sulfide is recognized.

Vaginal cultures of 20 ewes which gave birth to full-term lambs during an epizootic of vibriosis revealed that ewes which give birth to normal lambs may yield *V. fetus* when cultured shortly after lambing, and that *V. fetus* disappears from the vagina within a few weeks and is sometimes replaced by *V. bubulus*, which may remain in the vagina for a considerable length of time. *Vibrio bubulus* was also isolated after normal lambing from ewes which did not have any contact with aborting ewes.

Vibrio bubulus was isolated from the abomasum of a lamb born at full term which died shortly after birth, probably due to chilling.

A flock of 27 ewes, held during the winter and spring of 1955 and 1956 on a small, poorly drained pasture which had been heavily contaminated with *V. fetus* during a natural epizootic of vibriosis in the spring of 1955, had no evidence of infection with *V. fetus*.

In cross-agglutination studies, there was little relationship between *V. bubulus* and *V. fetus* strains of ovine and bovine origin. There were few cross-agglutination reactions within the *V. bubulus* group, indicating the existence of a number of serotypes.

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SUMMARIO IN INTERLINGUA

Le Isolation de *Vibrio bubulus* (Florent) ab Oves

Esseva obtenite culturas ab le muco preputial de 85 arietes in 12 ranchos e ab le semine de 33 arietes in 8 ranchos immediatemente post un epizootia de aborto vibrionic. *Vibrio fetus* non esseva isolate in ulle del casos, sed 40 culturas—positive a sulfuro de hydrogeno e negative a catalase e con leve grados de crescentia in culturas a punctura profunde—esseva isolate ab muco preputial. Dece-octo simile culturas esseva isolate ab semine. Omne le culturas tolerava 2,5% o plus de chloruro de natrium. Simile organismos esseva isolate ab le muco preputial e ab le semine de arietes in un grege sin recente antecedentes de aborto.

Super le base de tests physiologic, le culturas isolate ab arietes pareva esser identic con 4 racias de *V. bubulus* (Florent) de origine bovin que esseva obtenite ab Francia. Es pro-

ponite que vibriones de origine ovin que es physiologicamente comparabile con *V. bubulus* deberea portar ille nomine. Al tempore presente, le differentias inter *V. fetus* e *V. bubulus* pare sufficer pro justificar le stipulation de duo distincte species. Vibriones que produce catalase sed que alteremente ha le characteristicas de *V. bubulus*—tal como un tolerantia pro 2,5% o plus de chloruro de natrium e le capacitate de crescer in puncturas profunde—resimila *V. bubulus* plus proximemente que *V. fetus* e deberen esser placiate tentativemente in le prime del duo mentionate species. Le facto que cognoscite culturas de *V. fetus* pote producer micre quantitates de sulfuro de hydrogeno es recognoscite.

Culturas vaginal ab 20 oves feminin que parturiva agnos a termino normal durante un epizootia de vibriosis revelava que oves que produce agnos normal pote render *V. fetus* in culturationes effectuate brevemente post le parturition e que *V. fetus* dispare ab le vagina intra pauc septimanas e es reimplaciate a vices per *V. bubulus* que pote remaner in le vagina durante considerabile periodos de tempore. *V. bubulus* esseva etiam isolate post parturitiones normal ab oves que habeva habite nulle contacto con oves abortante.

V. bubulus esseva isolate ab le abomaso de un agno nascite a termino que moriva brevemente post nato, probabilemente a causa de frigidation.

Un grege de 27 oves feminin, mantenite durante le hiberno e le primavera de 1955 a 1956 in un micre e mal drainate pastura (que habeva essite fortemente contaminate con *V. fetus* durante un epizootia de vibriosis in le primavera de 1955), habeva nulle indication de infection con *V. fetus*.

In studios de agglutination cruciate, pauc relation se monstrava inter racias de *V. bubulus* e de *V. fetus* de origines ovin e bovin. Occurreva pauc reactiones de agglutination cruciate intra le gruppo de *V. bubu'us*, lo que indica le existentia de un numero de serotypos.

Fecal Elimination of Estrogens by Cattle Treated with Diethylstilbestrol and Hexestrol

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SYNTHETIC ESTROGENS, particularly diethylstilbestrol (stilbestrol), are used for estrogen therapy and also as growth promoters in avian, bovine, and ovine nutrition. Hence, information regarding the elimination of these compounds assumes more than academic interest.

The object of this study was to determine the effects of time, route of administration, dosage, and type of hormone on fecal elimination of estrogens.

In 1945, Levin [7] reported that fecal elimination of estrogens in cattle increased during pregnancy. El-Attar and Turner [4] later demonstrated that this estrogen excretion was maximal between gestation days 248 and 273. Fecal excretion of estrogen by stilbestrol-treated cattle was reported by Turner [13]; excretion by stilbestrol- and hexestrol-treated cattle, by Stob.[12] Reuber [10] studied fecal estrogenic elimination in heifers 60 days after initiation of oral treatment with stilbestrol. He reported a fecal excretion of about 44% of the estrogen consumed by heifers treated with 10 mg. and about 43% excretion of the total administered dose in heifers treated with 20 mg. of stilbestrol. Glascock and Hoekstra [6] showed that the subcutaneous injection of 25 μg. of tritium-labeled hexestrol (arachis oil vehicle) in kids or lambs resulted in an almost complete concentration of radioactivity in the urine, feces, and intestinal contents within 24 to 48 hours. Mitchell *et al.*[8] treated a steer with 100 mg. of tritium-labeled stilbestrol over an 11-day period and found 30% of the administered radioactivity in the feces and 20% in the urine. Ninety-eight per cent of the fecal estrogen was present in the free form, while 73% of the urinary estrogen was conjugated.

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MATERIALS AND METHODS

Fecal samples were obtained from beef-type heifers, 12 in each of 6 groups. The treatments were as follows: group 1, control; group 2, 10 mg. of stilbestrol orally per animal daily; groups 3 and 4, 24 and 36 mg. of stilbestrol, respectively, implanted subcutaneously; groups 5 and 6, 24 and 36 mg. of hexestrol, respectively, implanted subcutaneously. All heifers were given the same standard fattening ration. The subcutaneously administered estrogens were implanted at the base of the ear, while the hormone administered orally was incorporated in the protein supplement. Homogeneous fecal samples were taken from heifers of each group on days 3 and 7, and thereafter at weekly intervals for 24 weeks. Following collection, the samples were oven-dried at 100 C. for 24 hours, finely ground in a Wiley mill, and extracted. Extraction was effected by placing 90 Gm. of dried, ground, fecal material in 500 ml. of 95% ethanol and refluxing for 1 hour. The refluxed material was filtered under vacuum through No. 42 Whatman filter paper in a Buchner funnel. The filtrate was poured through the material and filtered a 2nd time, after which the refluxed material was rinsed with 100 ml. of 95% ethanol. The total filtrate was then poured over 70 Gm. of stock mouse ration and oven-dried at 100 C. This test ration was mixed in a Waring blendor, weighed, and brought up to 100 Gm. with additional stock ration in order to equate 1 Gm. of test ration with 1 Gm. of the original dried fecal sample.

Assay animals were mature, ovariectomized, female Rockland-Swiss mice. In order to allow for uterine atrophy, the mice were not used for assay purposes until 3 weeks following castration. Five mice were randomly assigned to each treatment; the control groups contained 10 mice each. Stock ration containing fecal extracts was fed to the mice at the rate of 3 Gm. per mouse daily for 6 days. Twenty-four hours after the last feeding, the mice were killed and their uteri dissected from surrounding tissue. The uterine horns were opened longitudinally and weighed on a torsion balance.

All data were analyzed statistically. The data were plotted arithmetically to show the relationship of time and fecal estrogenic activity and then were tested for linearity. Since a straight line fitted the data from all treatments, linear regression and correlation were computed in order to determine if there were significant trends in the various treatments. Linear lines were then fitted to data for treatments which were significant in

order to estimate where treatment data became equal to the control data with respect to elimination of fecal estrogens. Subsequently, confidence intervals for these point estimates were calculated. With these results, comparisons between treatments were made over the time during which the results of treatment were significantly different from the control results.

RESULTS AND DISCUSSION

Data collected from the assay of bovine fecal samples are presented (Table 1). Cattle treated orally with 10 mg. of stilbestrol daily excreted a uniformly significant ($P<0.01$) amount of estrogen in the feces throughout the 168-day treatment period. The fecal estrogen level was greater than that of the control or stilbestrol- or hexestrol-implanted cattle ($P<0.01$). Heifers subcutaneously treated with 24 and 36 mg. of stilbestrol excreted significant ($P<0.01$) amounts of estrogen until the 60th and 51st day, respectively. This period of significant fecal excretion parallels the 60- to 80-day period of maximal growth response of stilbestrol-treated heifers reported by Clegg and Carroll.[2] At no time was the fecal elimination of estrogen in the hexestrol-treated cattle greater than that of the controls. This may merely reflect the difference in degree of estrogenic potency between stilbestrol and hexestrol (Table 2). Preston *et al.*[9] state that, because of its

TABLE 1—Uterine Weight Response (mg.) of Mice to Extracts of Fecal Samples from Control and Hormone-Treated Cattle

Week No.	Heifer treatment					
	Control	Stilbestrol (10-mg., oral)	Stilbestrol (24-mg. implant)	Stilbestrol (36-mg. implant)	Hexestrol (24-mg. implant)	Hexestrol (36-mg. implant)
Day 3	33.36	128.84	50.76	36.68	27.64	31.08
1	25.00	135.16	50.84	64.52	35.08	32.96
2	24.68	146.64	41.00	66.48	24.96	22.32
3	23.56	142.32	28.12	24.24	22.44	24.24
4	22.04	140.16	60.55*	82.92	25.00	22.20
5	23.08	129.24	51.68	44.56	24.72	26.28
6	22.84	136.16	49.08	66.64	31.32	24.32
7	24.76	158.68	34.20	57.44	27.72	24.40
8	19.76	124.96	57.12	49.30*	22.64	27.48
9	24.26	133.04	31.60	47.80	30.68	26.04
10	21.32	122.50*	38.92	45.16	28.64	24.32
11	40.56	124.68	52.56	55.32	24.02	27.28
12	28.38	102.80	40.28	41.26	26.96	24.84
13	25.30	129.64	34.18	25.94	23.12	23.24
14	21.56	107.92	22.88	21.48	19.15*	20.84
15	27.56	166.64	34.48	27.56	28.52	32.12
16	25.96	141.72	41.95*	41.48	25.64	30.24
17	23.12	154.48	31.24	34.64	24.88	19.84
18	21.30*	169.12	25.56	25.10*	25.70	23.80
19	21.40	167.28	30.00	24.72	21.96	25.16
20	25.28	138.84	29.04	23.76	23.20	21.44
21	26.64	168.16	28.60	33.08	21.56	31.32
22	20.08	130.56	24.04	27.44	18.84	22.00
23	24.08	148.48	24.36	23.70*	21.88	25.64
24	23.40	148.04	22.40	14.20	17.76	18.04

Uterine weights listed are the means of 5 mice. * Means of 4 mice.

TABLE 2—Uterine Weight Response of Ovariectomized Mice to Orally Administered Stilbestrol and Hexestrol

Hormone in stock ration (μg./Gm.)	Stilbestrol		Hexestrol	
	Mean uterine weight (mg.)	Percentage of body weight	Mean uterine weight (mg.)	Percentage of body weight
0.01	25.22*	0.11	20.64	0.09
0.02	35.93*	0.16	.....	.....
0.04	67.22	0.30	23.74	0.10
0.08	98.56	0.42	30.76	0.14
0.16	119.70	0.51	38.36*	0.17
0.20	125.22	0.54	46.52	0.21
0.30	115.98	0.48	68.14	0.32
0.40	.....	.....	58.62*	0.24
0.80	.....	.....	76.90*	0.34

Uterine weights listed are the means of 10 mice. * Means of 9 mice.

reduced estrogenic potency in man, hexestrol is the preferred hormone for cattle-feeding purposes in the United Kingdom.

Swine following hormone-treated cattle may be adversely affected by consumption of estrogenic fecal material. Suppression of testicular development in young boars exposed to hormones in this manner was reported by Stob.[12] Stevenson and Ellis [11] have shown, however, that gilts 4½ months of age or older, fed up to 25 mg. of stilbestrol daily for 76 days, are rendered sterile only temporarily and are restored to normal reproductive function upon withdrawal of the estrogen.

Hertz (Gassner *et al.*[5]) has posed the possibility of contamination of water supplies and hormone uptake by plants fertilized with estrogenic fecal material. It is well known that certain plants are estrogenic,[1] but it is not known if plant roots or leaves are permeable to estrogen molecules. The water solubility of hormones would be an important factor in either water supply contamination or plant uptake. Dodds [3] has reported that for stilbestrol the degree of water solubility is very low, *i.e.*, 4 to 5 mg. per liter.

SUMMARY

The estrogenic activity of the feces of estrogen-treated and control heifers in 6 groups was determined during a 24-week period following initiation of hormone treatment. Animals given 10 mg. of diethylstilbestrol orally per day excreted significantly more estrogen than did the controls or heifers subcutaneously treated with 24 or 36 mg. of either diethylstilbestrol or hexestrol. This excretion was uniformly high for the entire experimental period. Subcutaneous implantation with 36 mg. of diethylstilbestrol resulted in a greater total estrogen excretion than treatment with 24 mg. However, significant amounts of estrogen were excreted for 60 days following treatment with 24 mg. of diethylstilbestrol when compared to that excreted for 51 days following 36-mg. implantations; after this time, fecal estrogen elimination of heifers in both groups was similar to the controls. Implantation of heifers with 24 or 36 mg. of hexestrol did not result in greater fecal estrogen elimination than in the animals not given supplementary estrogen; neither treatment produced significant weekly variation in estrogen excretion.

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SUMMARIO IN INTERLINGUA

Le Elimination Fecal de Estrogenos per Bestial Tractate con Diethylstilbestrol e Hexestrol

Le activitate estrogenic del feces de cinque gruppos de vaccas virgine tractate con estrogeno e de un gruppo de controlo esseva determinate durante un periodo de 24 septimanas a partir del initiation del tractamento hormonal. Animales que recipeva 10 mg de diethylstilbestrol per die per via oral excerneva significativemente plus estrogeno que le animales de controlo e etiam que illos tractate per via subcutanee con 24 o 36 mg de diethylstilbestrol o

de hexestrol. Iste excretion esseva uniformemente alte durante le complete periodo experimental. Le implantation subcutanee de 36 mg de diethylstilbestrol resultava in un plus grande excretion de estrogeno total que le tractamento con 24 mg. Tamen, quantitates significative de estrogeno esseva secernite durante 60 dies post le periodo del tractamento con 24 mg de diethylstilbestrol in comparation con le quantitates excernite durante 51 dies post le implantation de 36 mg. Post iste tempore, le elimination fecal de estrogeno in ambe le mentionate gruppos esseva simile a illo del gruppo de controlo. Le implantation de 24 o 36 mg de hexestrol in le vaccas virgine non resultava in plus alte concentrationes del elimination fecal de estrogeno que in le animales non tractate con estrogeno supplementari. Le animales in ambe gruppos non habeva significative variationes septimanal del excretion de estrogeno.

White Muscle Disease (Myopathy) in Lambs and Calves
VII. Etiology and Prophylaxis

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IT HAS previously been shown that the administration of selenium gives a high degree of protection against white muscle disease (WMD) in lambs and calves.[4, 15, 16, 20, 23] Some of these results have also demonstrated that treatment with Se resulted in marked body weight gains in the young.[5, 8, 12, 18, 26] These findings have suggested the possibility of a Se deficiency or of some substance interfering with Se metabolism in the feeds associated with this myopathy.

It has also been reported that vitamin E is of value in treating young lambs affected with WMD,[1, 9, 11, 27, 30] although it is of no apparent value when administered to the pregnant ewe [14, 16] in reasonable amounts.

Field investigation [10, 28] and feeding trials [22, 29] have indicated that some concentrates are apparently protective against WMD when fed to the pregnant females but whether this is due to available Se, factor 3,[21] or some other factor has not been reported.

There is evidence that sulfates interfere with Se uptake by plants [8] and that they alleviate Se toxicity in microorganisms [2] and in rats.[6] Also in the experimental production of WMD in calves,[17] a regressive incidence of WMD was obtained in the calves when succeeding years' crops of alfalfa from the same planting were fed to cows. This has since been associated with a decline from high inorganic and total sulfur contents in the forage in the face of apparently adequate Se contents over a 3-year period.[13] Therefore, the question naturally arises as to the role of sulfur in other biologic processes involving Se, including possible similar interference in the alimentary tract or elsewhere in animals, particularly ruminants.

The trials reported herein were designed to study further the effect of Se and vitamin E on lambs, and to study other aspects of the disease.

MATERIALS AND METHODS

Seven lots of 12 ewes each were used in these experiments. They were similar to and maintained like those in previously reported trials.[16] Those in lot 1 were given the basal ration only, consisting of 4 lb. Ladino hay and 0.25 lb. ground oats/ewe/day. Those in lot 2 were given the basal ration and the lambs were given 0.28 mg. Se as Na_2SeO_3 subcutaneously in physiologic saline the day of birth, followed by 4 times that amount (1.12 mg. Se) at 2 weeks of age. This was calculated to be 0.2 p.p.m. of the assumed dry matter intake during the first 6 weeks of life.

Ewes in lot 3 were given the basal ration and the lambs were given 1,000 I.U. α-tocopherol* orally the first day of life followed by a like amount on the second day. Ewes in lot 4 were fed the basal ration supplemented with 0.1 p.p.m. Se calculated on a dry-matter basis, as Na_2SeO_3 added to the ground oats. Ewes in lot 5 were fed the basal ration plus 0.1 p.p.m. Se and Na_2SO_4 mixed with the ground oats, starting at the rate of 0.059% of the entire ration and increasing the amount until a calculated level of 0.236% was reached for approximately the last 30 days of the regimen. This gave a calculated total of 0.216% sulfur of the entire ration (*see* Table 1), thereby approximating the level of inorganic and total sulfur previously encountered in a severely myopathogenic alfalfa hay.[13]

In lots 6 and 7, 0.25 lb./ewe/day of soybean oilmeal and linseed oilmeal,** respectively, were substituted for the ground oats of the basal ration. The experimental regimens were started when the ewes were about 2 months pregnant and continued for approximately 140 days.

All lambs were weighed at birth, at 2 weeks, and at 6 weeks of age when those surviving were killed and examined at necropsy for the presence of gross lesions. Blocks of tissue were routinely

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TABLE 1—Sulfur in Ration of Ewes in Lot 5

Component	Per cent of ration	
	Inorganic S	Total S
Forage calculated at 4 lb	0.030	0.151
Oats calculated at 0.25 lb	nil	0.012
Sodium sulfate	0.053	0.053
Total	0.083	0.216

selected, processed, and examined as previously described.[26]

Blood tocopherol levels were determined in 6 ewes from lots 1 to 5 inclusive at the beginning of the feeding period, at midpregnancy, and just before lambing. The blood tocopherol levels of these ewes' lambs were also determined when the lambs were killed at 6 weeks of age, and the dialuric acid erythrocyte hemolysis test (a positive reaction to which indicates a vitamin E deficiency in some species)[2] was applied to all blood samples. The forage fed was analyzed for dry matter, crude fat, crude fiber, crude protein, calcium, phosphorus, sulfur, and selenium. The oats and oil meals were analyzed for calcium, phosphorus, magnesium, potassium, and sulfur.

In addition to the animal experiment reported herein, forage samples were collected from 16 ranches where WMD is common and 3 where it had not been experienced and these samples were subjected to selenium analysis.*

treatment used in lot 4 was most highly effective in the prevention of WMD (as expressed by the occurrence of lesions and weight differences). When Na_2SO_4 was superimposed on the Se treatment (lot 5), it apparently interfered with the protective action of Se (lot 4).

Data concerning blood tocopherol analyses show that the lambs in lot 3, given massive doses of α-tocopherol during the first 2 days of life, had significantly higher blood tocopherol levels than did the lambs in all the other lots which had received no vitamin E. However, none of the blood samples tested, including all lamb and ewe samples collected, were positive to the dialuric acid erythrocyte hemolysis test. Analysis of the experimental forage revealed a Se content of the order of 0.05 p.p.m.* Values obtained for the other elements appear to be within normal limits.

Values for Se in 29 samples of legumes and mixed forages obtained from 16 affected ranches range from 0.06 to 1.30 with an average of 0.67 p.p.m. Selenium values for 9 similar samples from 3 ranches where WMD has not been a problem ranged from 0.18 to 2.80 with a mean of 0.54 p.p.m.

TABLE 2—WMD Lesions and Weight Gains in Lambs on Various Treatments

	Lot treatment	No. of lambs	Gross lesions	Micro lesions	Micro lesions calcified	Wt. gain birth to 6 weeks
1	Basal ration to ewes only	20	16	19	11	15.32(18)*
2	Basal ration to ewes and Se subcutaneously to lambs	17	0	7	0	20.84(14)
3	Basal ration to ewes and vitamin E to lambs	17	0	5	0	19.28(15)
4	Basal ration plus 0.1 p.p.m. Se to ewes	18	0	3	0	27.15(10)
5	Basal ration plus 0.1 p.p.m. Se plus Na_2SO_4 to ewes	14	1	9	1	21.61(14)
6	Basal hay plus soybean oil meal to ewes	16	0	5	0	23.61(16)
7	Basal hay plus linseed oil meal to ewes	17	0	5	0	25.79(16)

* Number of surviving lambs killed at 6 weeks of age.

A statistical analysis of the data contained in Table 2 shows that treatments used in lots 2 to 7 inclusive were all significantly effective, both in reducing the incidence of lesions and in increasing weight gains when compared with lot 1, in which the ewes received the basal ration only ($P < 0.05$). It also indicates that the Se

DISCUSSION

It is apparent from the results obtained that not only was the treatment of sheep in lot 1 conducive to a high incidence of WMD, but that treatments superimposed on the same basal diet in the other lots all had a marked influence on the course of the disease. There was a significant difference be-

* Kenneth C. Beeson, director, ARS, Plant, Soil and Nutrition Laboratory, Ithaca, N.Y., collected and analyzed these samples.

* P. R. Stout, director, Kearny Foundation for Soil Science, University of California, Berkeley, provided this Se analysis.

tween the results obtained in lot 1, where the ewes were given the basal myopathogenic ration only, when compared with lot 2 in which the lambs were given small injections of Se. There was also a significant difference between the average weight gains in this lot and lot 4, indicating that the hypodermic treatment at the dosage level employed in lot 2 was not so effective as the feeding of Se to the ewes in lot 4. It was not surprising that massive doses of vitamin E as administered to the lambs in lot 3 would have a therapeutic effect, since the pharmacodynamic relationship of vitamin E and selenium has been well demonstrated.[24,25] It was interesting, however, that in the case of previous experiments,[14,16] α-tocopherol was not effective when fed to the ewes. This difference may be explained on the basis of the relatively inefficient maternal transfer of vitamin E [19] and the relatively large doses of that vitamin that may be necessary for the therapeutic effect under conditions similar to those encountered in these and previously reported experiments.[14,16]

Completely negative results to the dialuric acid erythrocyte hemolysis test indicated that, by this criterion, none of the animals were particularly depleted of vitamin E, if this is a valid test in this species.

The mechanism of the apparent interference resulting from the inclusion of Na_2SO_4 in the ration of sheep in lot 5 can only be postulated at this time. Actually, the interference may have been more significant than the table shows, since a progressive incidence of WMD in lambs born in this lot paralleled the S increase in the ration. This interference probably resulted because of the close chemical relationship of Se and S. Whether S in the feed plays a principal role in the cause of WMD remains to be determined, but when the apparently adequate Se content of other offending forages surveyed are considered, it appears that S and perhaps other substances interfere with the metabolism of Se, and that this interference results in WMD and suboptimal weight gains or both.

While the soybean and linseed oil meals both gave a high degree of protection against WMD under the conditions of these trials, their mode of action likewise can only be postulated. In view of the apparently adequate Se values in all feeds examined, it would appear that sufficient Se in some form to meet normal demands is available in these meals or that some other presently unrecognized substance present is capable of protection against WMD.

The demonstration of apparently adequate, but unavailable, Se in feeds which, when fed pregnant ewes, results in WMD in their young, opens a new vista of this problem which should point to a solution of the basic cause and a better understanding of the metabolic processes involved.

SUMMARY AND CONCLUSIONS

Pregnant ewes in 7 lots of 12 each were fed a basal myopathogenic ration of 4 lb. of Ladino clover hay plus 0.25 lb. of oats/ewe/day. In addition, the various lots of ewes were given the following treatment: (1) none; (2) selenium subcutaneously to the lambs; (3) vitamin E orally to the lambs; (4) 0.1 p.p.m. Se to the ewes in feed; (5) 0.1 p.p.m. Se plus Na_2SO_4 to supply 0.216% S in feed to the ewes; (6) soybean oil meal substituted for oats in ewes' ration; (7) linseed oil meal substituted for oats in ewes' ration. All treatments gave some degree of protection, with Se in ration of ewes ranking highest, but there was evidence that where Se and Na_2SO_4 were given, the sulfate interfered with the action of the Se. Lambs given massive doses of α-tocopherol at birth had significantly higher blood tocopherol values at 6 weeks than did untreated lambs. Analysis of 29 samples of legumes and mixed forages from 16 affected ranches revealed Se values ranging from 0.06 to 1.30 p.p.m. It therefore appears that, on the basis of present information, the disease as it occurs in this area is not primarily a Se deficiency but results from substances (possibly including sulfur) causing an aberrant metabolism of the Se present in the feeds.

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SUMMARIO IN INTERLINGUA

Morbo de Musculo Blanc (Myopathia) in Agnos e Vitellos
VII. Etiologia e Prophylaxe

Oves pregnante in 7 gruppos de 12 cata un recipeva un dieta myopathogene basal de 4 libras de feno de trifolio ladin con 0,25 libras de avena per capite per die. In plus, le 7 gruppos esseva tractate secundo le sequente schema: Prime gruppo, nulle tractamento special; secunde gruppo, selenium per via subcutanee in le agnos; tertie gruppo, vitamina E per via oral in le agnos; quarte gruppo, 0,1 partes per million de selenium in le dieta del oves; quinte gruppo, 0,1 partes per million de selenium e un quantitate de Na_2SO_4 sufficiente a representar un contento de 0,216 pro cento de sulfure in le dieta del oves; sexte gruppo, pasta residue de oleo de soja como reimplaciamento de avena in le dieta del oves; septime gruppo, pasta residue de oleo de lino como reimplaciamento de avena in le dieta del oves. Omne iste formas de tractamento provideva un certe grado de protection. Isto esseva le plus marcate in le caso de selenium in le dieta del oves, sed certe observationes indicava que in le combination de selenium con Na_2SO_4, le sulfato interfereva in le action del selenium. Agnos tractate al nascentia con massive doses de alpha-tocopherol habeva significativemente plus alte nivellos de tocopherol sanguinee al fin de 6 septimanas que le agnos non assi tractate. Le analyse de 29 specimens de legumine e de forrage mixte ab 16 afficite ranchos revelava valores pro selenium de inter 0,06 e 1,30 partes per million. Assi il pare, super le base del presente information, que le morbo como illo occurre in iste area non es primarimente causate per un carentia de selenium sed resulta ab substantias (possibilemente con le inclusion de sulfure) que induce un aberrante metabolismo del selenium presente in le forrage.

Studies on Shipping Fever of Cattle. II. Prevalence of Pasteurella Species in Nasal Secretions from Normal Calves and Calves with Shipping Fever

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PASTEURELLA species are commonly found in cattle with respiratory infections. Their role, however, has not been clearly established. In 1895, Moore [10] reported *Pasteurella spp.* to be commensals on the respiratory mucous membranes of normal animals. Jorgensen [8] confirmed these findings in his study of 250 cattle and concluded that *Pasteurella boviseptica* produced disease only after the natural resistance of the host was lowered due to cold or fatigue. He was unable to distinguish the bacteria which were present in diseased animals from those found in normal animals.

Scott and Farley [12] isolated *Past. boviseptica* from 80% of the cases of shipping fever studied. Carter [1, 2] reported bronchopneumonia in calves from 4 to 10 months of age to be caused by pasteurellas and showed that *Pasteurella spp.* were also the predominant bacteria found in cattle with shipping fever. Carter and McSherry [4] examined nasal swabs from 33 cattle with shipping fever and concluded that this disease was a pasteurellosis. Carter,[3] in 1956, produced mild-to-severe infection in calves by artificially exposing them to *Pasteurella hemolytica,* but most other workers have had little success with the artificial production of disease with these organisms.

The use of biological products specific for the pasteurellas has not been highly successful in the prevention or treatment of shipping fever. However, the fact that the condition responds to treatment with sulfonamides and antibiotics is strong evidence that the signs of disease are caused by bacteria.

Shipping fever seems to follow lowered resistance of the host by physical environmental factors such as cold; abnormal physiologic factors such as fatigue, change of feed and water; and is possibly even influenced by factors such as fright and anxiety.[6] Evidence of predisposing viral infections has been found recently.[6, 7, 9, 11]

This report presents some of the studies on the pasteurellas found during the course of investigations into the pathogenesis of shipping fever.

MATERIALS AND METHODS

Groups of beef calves, 6 to 10 months of age, were studied in several different Illinois feedlots during the fall of 1958. These calves had originated from different areas and were handled by several commonly accepted systems of management after arrival in the feedlots.

One group of calves consisted of 25% of the animals consigned to a feeder calf sale from 27 different herds in southern Illinois. Nasal swabs were procured from these 200 calves as they arrived at the sale. After sampling, the calves were sorted into various sale lots according to weight, grade, and sex so that animals from the different herds were mixed before being sold. After the sale, many of these calves were studied following shipment to 7 feedlots in central and northern Illinois.

In one of these feedlots, calves from the sale were added to a larger group of "sale barn" calves from another source. Fifteen days after the sale, approximately 75% of the more than 300 calves in the feedlot either had signs of shipping fever or were febrile and were treated. Nasal swabs were studied from 35 of the calves first sampled on arrival at the feeder calf sale.

In another feedlot, a severe epizootic of shipping fever occurred in calves purchased at the feeder calf sale. When observed 19 days after the sale, many of these calves had been subjected to intensive therapy. Nasal swabs were obtained from 22 calves treated with several injections of penicillin and streptomycin.

A 2nd nasal swab was obtained from 109 of the calves which had been first sampled at the feeder calf sale, after they had been in the 7 feedlots under study for 4 to 7 weeks. All of these feedlot calves had had mild to severe shipping

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fever. When the second nasal swab was taken, most of the calves had recovered but some still had effects of the disease. While these nasal swabs are referred to as "convalescent," many of these calves had no apparent signs of shipping fever during the interim between nasal samplings.

Fifty calves raised in Texas arrived at the Animal Science Division feedlots, University of Illinois, late in August. Body temperatures of these calves were taken daily following arrival. During the first 18 days, nasal swabs were taken from 29 calves with temperatures over 104 F. but with no other signs of disease. Clinical signs of shipping fever developed after the calves had been in the feedlot 20 days, and nasal swabs were collected from 17 of the affected animals.

At the Dixon Springs Experiment Station, University of Illinois, 233 calves were studied for evidence of respiratory disease following weaning. Nasal swabs were collected from 43 calves with body temperatures over 104 F. during the 11 days following weaning. The fever was transient and was not accompanied by other signs of disease. Respiratory disease did not develop in these calves.

The nasal secretions of the calves included in these studies were collected by means of sterile cotton swabs applied to the mucous membranes of one side of the nasal cavity, care being taken to avoid contact with the skin of the anterior nares. The swabs were streaked on 10% horse blood tryptose (Bacto) agar plates. After incubation of the plates for 18 hours at 37 C., colonies suspected of being *Pasteurella spp.* were subcultured for identification by differential sugar fermentations. Other bacteria were also studied in some series of plates.

Results

During these studies, 455 nasal swabs were examined. From the 200 swabs from normal calves, only 6 cultures of Pasteurella spp. were recovered (Table 1). However, 31 (59.6%) of the 52 calves with clinical shipping fever had pasteurellas in their nasal secretions.

Pasteurella organisms occurred less frequently in the calves given intensive antibiotic treatment. Many of the swabs from these calves yielded sterile plates.

Calves with fever but without other signs of disease had pasteurellas more frequently than the normal calves, but the bacteria were not so prevalent as in those having clinical shipping fever.

Pasteurellas were found in 39.4% of the calves from the groups convalescent from shipping fever.

Of 203 nasal swabs examined for hemolytic cocci in addition to pasteurellas, 72 (35.4%) were found to contain hemolytic streptococci. Of the 455 swabs studied, 24 (5.2%) showed the presence of *Alcaligenes spp.*

Discussion

The low incidence of pasteurellas in the group of apparently normal calves may have been due to the fact that most of the calves were free of these bacteria or that the number of organisms was so small that they were not detected by the sampling methods used. The calves which later had signs of shipping fever and positive cultures of *Pasteurella spp.* may have had only an infinitesimal number of organisms when first sampled, but when the resistance of the nasal mucous membrane became lowered these bacteria were able to multiply. It is also possible that the organisms carried by an apparently normal calf are prevented from multiplying by immune mechanisms and that the strains recovered during respiratory disease are different strains picked up from the other calves in the feedlot. The opportunity for spread of various strains of Pasteurella is certainly present under feedlot conditions. It may be possible to obtain answers to these problems through lysogenicity studies.[12] By use of the different phage types, the transfer of pasteurellas from animal to animal could be determined.

It could not be determined from these studies whether pasteurellas were responsible for the observed signs of disease. The lower prevalence of pasteurellas in the nasal secretions of calves being treated with antibiotics may be related to the generally favorable results following therapy. Regardless of the exact pathogenesis of shipping fever, once the disease is established,

TABLE 1—The Occurrence of Pasteurella spp. in Calves

History of animals	Nasal swabs examined	Pasteurella spp. isolated	Per cent positive
Apparently normal	200	6	3
Fever of 104 F. (no other signs of disease)	72	15	21
Clinical shipping fever	52	31	60
Clinical shipping fever with intensive antibiotic treatment	22	3	14
Convalescent from shipping fever	109	43	40

the control of bacteria appears to be important to recovery. Concurrent infection with myxovirus parainfluenza 3 was demonstrated in all of these groups of calves.[7]

The significance of the presence of hemolytic streptococci found in nasal swabs from cattle with shipping fever should be investigated.

SUMMARY

Nasal swabs from apparently normal calves and from calves with different stages of shipping fever were examined for the presence of *Pasteurella spp.*

Pasteurellas were found in only 3.0% of 200 normal calves, but in 59.6% of those with clinical shipping fever.

Of the calves with shipping fever which were given intensive antibiotic therapy, only 13.6% harbored pasteurellas; 20.8% of calves with fever as the only sign of disease had *Pasteurella spp.* Of 109 nasal swabs examined from groups of calves "convalescent" from shipping fever, 43 (39.4%) contained pasteurellas.

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SUMMARIO IN INTERLINGUA

Studios Relative a "Febre de Transporto" in Bestial. II. Prevalentia de Species de Pasteurella in le Secretiones Nasal de Vitellos Normal e de Vitellos con Febre de Transporto

Tampons nasal ab apparentemente normal vitellos e ab vitellos in varie stadios de febre de transporto esseva examinate pro le presentia de species de Pasteurella.

Pasteurellas esseva trovate in solmente 3,0 pro cento de 200 vitellos normal sed in 59,6 pro cento del vitellos con febre de transporto de grados clinicamente significative.

Inter vitellos con febre de transporto tractate per intense therapias antibiotic, solmente 13,6 pro cento revelava le presentia de pasteurellas; 20,8 pro cento del vitellos con febre como le sol signo de morbiditate habeva species de Pasteurella. Inter 109 tampons nasal obtenite ab vitellos in reconvalescentia ab febre de transporto, 43 (= 39,4 pro cento) contineva pasteurellas.

Bovine Papular Stomatitis. II. The Experimentally Produced Disease

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THE DISCOVERY of naturally occurring bovine papular stomatitis in Ohio [1] prompted this study of the experimentally produced disease. In addition to confirming the diagnosis, reproduction of papular stomatitis in the laboratory provided additional, more accurate characterization of the disease as well as material for subsequent histopathologic study.[2] The pathogenicity of the causative virus for mice, guinea pigs, embryonating eggs, and tissue cultures was also investigated.

MATERIALS AND METHODS

The 44 calves employed in this study ranged from 1 week to 4 months of age. The herds from which these calves were selected were known to have been healthy (except for mastitis and occasional calf scours) for at least the 2 preceding years. As additional controls, all the calves in each herd were examined for the presence of stomatitis and rhinitis at the beginning and end of each experiment. The calves were housed in isolation rooms which, except for 2 preliminary experiments, had never contained cattle. Powdered milk substitute was fed in place of whole milk. Baseline studies were conducted for 1 to 3 weeks at the beginning of each experiment. Daily procedures consisted of determination of the total circulating and differential leukocyte counts and a general physical examination, including appetite, excreta, pulse, respiratory rate, rectal temperature, and auscultation of heart, lungs, and rumen.

Determinations of body weight, hemoglobin, sedimentation rate, and total erythrocyte count were made every 4 days. Ophthalmoscopic examination, fecal flotation, complete urinalysis, and the collection of serum samples were all performed at least once before inoculation and several times during the experimental period. During the first 3 days after inoculation, total circulating and differential leukocyte counts were performed every 12 hours and rectal temperatures were taken every 4 hours.

The source of the original tissues infected with bovine papular stomatitis was 2 naturally infected calves. The original virus was isolated from calf B426 in bovine kidney cell culture. Tissue suspensions and chicken embryo-passaged material came originally from calf B426.

Tissue cultures were prepared by a method similar to that of Madin *et al.*[3] Stationary trypsinized cultures were grown on glass in a nutrient medium containing lactalbumin hydrolysate, Tris buffer,* and either pooled ovine serum or plasma from a newborn calf. Half of the cultures were grown on coverslips which were removed, fixed in Bouin's fluid, and stained by either hematoxylin and eosin or May-Grünwald-Giemsa technique.

The mice (BALB/c) used in this study were from a well-isolated colony in the 69th generation of brother-sister matings. Many serial passages of brain intracerebrally and lung intranasally had been made in mice from this colony with no evidence of disease. In every experiment using laboratory animals or tissue cultures, parallel control serial passages were made starting with uninoculated animals or cultures. In each experiment, microscopic examination was made of randomly selected laboratory animals or embryonating eggs.

All materials suspected of containing viruses were stored at −65 C. Infected tissues were ground in phosphate-buffered saline or tissue culture nutrient medium containing 2% serum before freezing. Glycerol was not used.

RESULTS

Bovine papular stomatitis was reproduced in 10 of 15 experimental calves; 32 controls remained free of infection. The virus of bovine papular stomatitis was successfully transmitted to 3 calves by tissue culture material, to 3 calves by chicken embryo chorioallantoic membranes, to 1 calf by a swab from a lip lesion, to 2 calves by suspension of lip and palate lesions, and to 1 calf placed in a pen with a naturally infected calf. Suspensions of internal organs (brain, lungs, liver, mesenteric lymph nodes, and kidneys) from a calf with a slight naturally occurring infection did not transmit papular stomatitis to any of the 5 calves exposed to this material.

All of the calves exposed in any way to lip lesions or virus propagated in tissue culture or chicken embryos developed papular

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* Tris buffer (Sigma 7-9) obtained from Sigma Chemical Co., St. Louis, Mo.

TABLE 1—Experimental Transmission of Bovine Papular Stomatitis to Young Calves

Calf No.	Type of exposure	Degree of infection
B638	Placed in pen with calf B426	Moderate
B168	Suspension of brain and lung from calf B167	None
B777	Suspension of brain from calf B167	None
B782	Suspension of viscera from calf B167	None
B778	Seitz filtrate of viscera from calf B167	None
B779	Seitz filtrate of brain from calf B167	None
B780	Control—formalin-inactivated viscera from calf B167	None
B781	Control—uninoculated	None
B777*	Suspension of lip and palate from calf B426	Severe
B779*	Suspension of lip and palate from calf B426	Moderate
B778*	Swab of lip lesion from calf B777	Moderate
B427	Chicken embryo-passaged material from calf B167	Moderate
B428	Chicken embryo-passaged material from calf B167	Moderate
B429	Chicken embryo-passaged material from calf B167	Slight
B1685	Tissue culture-passaged material from calf B426	Moderate
B1686	Tissue culture-passaged material from calf B426	Moderate
B1688	Seitz filtrate of tissue culture-passaged material	Moderate
B1687	Control—uninfected tissue culture medium	None
.....	Controls—9 uninoculated calves	None
.....	20 controls—scarification of oral mucosa without virus	None

* Not infected by previous exposure.

stomatitis. The clinical data, such as body weight, appetite, pulse, respiratory rate and body temperature, on all the experimentally infected calves, did not change appreciably from the baseline findings. Likewise, laboratory results including hemoglobin values, sedimentation rate, total leukocyte and erythrocyte counts, and urinalysis remained within the preinfection range during the entire observation period. Since the laboratory data did not deviate significantly from normal, they confirmed the effectiveness of isolation in preventing the occurrence of any disease other than papular stomatitis. Definitive antemortem detection of infection was necessarily based upon the appearance of papules in the mouth, pharynx, nostrils, muzzle, and skin around the mouth.

The type of exposure and the course of the experimental disease are summarized (Tables 1, 2).

Direct Contact.—On the 7th day after the appearance of lesions in a naturally infected animal (B426), calf B638 was placed in the same pen. Lesions of papular stomatitis appeared in the latter animal on the 3rd day after exposure, and a few lesions were still present when euthanasia was performed on the 98th day of observation.

Direct Transmission (Cortisone Preparation).—One experimental calf which was inoculated with fresh material was a 4-month-old male Jersey (B168) that had been prepared by daily administration of 200 mg. of cortisone acetate in 2 divided doses for 2 weeks prior to inoculation; 0.5 ml. of a fresh 20% suspension, in nutrient medium, of brain from calf B167 was inoculated intracerebrally and 20 ml. of the same suspension was instilled by the intranasal, oral, and conjunctival sac routes. Nine days later, the same calf was inoculated intramuscularly with 0.5 ml. of a 20% suspension of lung from calf B167. Cortisone acetate administration was continued but no signs of disease were detected after 16 days.

Direct Transmission (Inoculum—Internal Viscera Without Lesions).—Four calves, 2 to 4 weeks old, were inoculated with suspensions of tissues from calf B167. Inoculums were prepared from 20% suspensions of brain, lungs, liver, mesenteric lymph nodes, and kidneys that had been stored at −65 C. for 38 days. Calf B777 was inoculated intracerebrally with 0.25 ml. of a 1:5 suspension of brain. Calf B778 was given 20 ml. of a 1:100 Seitz ST-1 filtrate of pooled suspensions of viscera via intranasal, intravenous, intramuscular, conjunctival sac,

TABLE 2—Incubation Period and Course of Experimental Papular Stomatitis

Calf No.	Type of transmission	Incubation	Secondary lesions*	First regression	Duration
B638	Direct contact	3**	5**	19**	98**
B777	Direct transmission	5	7	14	97
B799	Direct transmission	3	5	....	6
B778	Direct transmission	5	6	18	78
B427	Indirect (chicken embryo)	9	11	12	15
B428	Indirect (chicken embryo)	6	....	10	28
B429	Indirect (chicken embryo)	11	....	....	18
B1685	Indirect (tissue culture)	2	4	3	16
B1686	Indirect (tissue culture)	3	5	14	92
B1688	Indirect (tissue culture)	2	8	8	13

* Lesions in addition to those at the site of inoculation. ** Days after inoculation.

and scarified lip and tongue routes. Calf B779 was inoculated intracerebrally similarly to calf B777 but with a Seitz filtrate. Calf B782 was inoculated via several routes similar to B778 but with a nonfiltered pool of the visceral suspensions. Control calf B780 was given 1.0 ml. intravenously and 1.0 ml. intramuscularly of a filtrate of pooled viscera mixed with 0.5% formalin for 45 minutes before inoculation. Calf B781 served as uninoculated control calf. There was no evidence of papular stomatitis after 63 days in the inoculated or control calves.

Direct Transmission (Inoculum—Lip and Palate with Lesions).—Calves B777 and B779 were each given, via intranasal, oral, conjunctival sac, and scarified oral mucosal routes, a total of 10.0 ml. of a 2% suspension of pooled lower lip and hard palate from calf B426. The inoculum had been stored at −65 C. for 76 days. A lesion of papular stomatitis appeared at the scarification site on the inner surface of the lower lip of calf B777 on the 5th day after inoculation. It started as a circular, reddened area with a roughened, raised, pale center, and gradually enlarged until the 21st day after inoculation when it appeared as a gray, flat, papillomatous plaque, 2.0 by 1.5 cm. and raised 2.0 to 4.0 mm. above the surface (Fig. 1, 2).

Secondary lesions were first observed on the 7th day and during the course of the disease appeared on the upper and lower lips, gingiva, dental plate, floor of the mouth, ventral tip of the tongue, dental pad, hard palate, muzzle, nostrils, and skin of the lower lip, chin (Fig. 3), and lumbar area.

On the 17th day after inoculation, biopsy was made of a papular lesion on the edge of the lower lip. Culture of a triturated portion revealed a facultative, *alpha*-hemolytic Streptococcus and an unidentified aerobic, gram-negative rod, neither of which was considered a pathogen.* The lesions regressed on the 14th day and again on the 28th day after inoculation, but a few lesions in various states of development were present until the 97th day. A wire suture was purposely left at the biopsy site to cause irritation, but no new lesions of papular stomatitis developed there. No lesions were found when euthanasia was performed on the 119th day.

A typical lesion of papular stomatitis appeared on the scarification site in the lower lip of calf B779 on the 3rd day after inoculation. Secondary lesions appeared the 5th day on the muzzle and hard palate, and the next day euthanasia was performed to obtain tissues for histopathology. Additional microscopic lesions were found in papillae on the dorsum of the tongue, buccal papillae, reticulum, and omasum.

Direct Transmission (Nasal Abrasion with Swab of Lesion).—Ten days after inoculation of calf B777, B778 was inoculated by the introduction of a fresh swab from the primary lesion on the lip of calf B777 into an abraded area on the ventral margin of one nostril. Cortisone acetate administration, 100 mg. twice a day intramuscularly, was started the day before inoculation and continued for 21 days. Lesions appeared at the inoculation site on the 5th day after inoculation, in the other nostril on the 6th day, on the muzzle and skin around the mouth on the 11th day (Fig. 4), and on the hard palate, gingiva, and dental plate on the 16th day. The primary lesion reached a size of 1.5 by 1.0 cm. just before all the lesions began to regress on the 18th day. Remnants of lesions disappeared on the 43rd day. A few new lesions were present

Fig. 1—Early proliferative lesion on the inner surface of the lower lip and gingiva of calf B777, 7 days after inoculation.

* Performed by Dr. E. H. Bohl, Department of Bacteriology, the Ohio State University, Columbus.

Fig. 2—Large proliferative lesion on the lower lip of calf B777, 21 days after inoculation—compare with Figure 1. Notice the thickening of the lip margin and the lesions on the gingiva (arrow) and upper lip.

between the 59th and 78th days, but no significant macroscopic lesions were found when euthanasia was performed on the 109th day. Cortisone acetate, which was continued until after the lesions had started to regress, had no apparent effect on the course of the disease.

Egg-Passaged Material.—Three Guernsey calves (B427, B428, and B429), 1 to 4 months old, were inoculated with egg-passaged materials. The inoculum was a suspension of pooled chorioallantoic membranes from the 2nd, 3rd, and 4th passages in embryonating eggs that originated with lungs, brain, and spleen from calf B167. They had been stored at −65 C. for 2 to 4 weeks. No evidence of disease had been detected in the chicken embryos but the possibility that a virus had survived or propagated could not be excluded.

Each of the 3 calves was inoculated with 2.0 ml. of a 10% suspension intravenously and 2.0 ml. intramuscularly; 10.0 ml. of a 1:100 suspension was instilled intranasally, 9.0 ml. orally, 0.5 ml. via conjunctival sac, and a few drops on small scarified areas of the buccal mucosa and ventral portion of the tongue. Calf B429 was given, intramuscularly, 25 mg. of prednisolone twice daily beginning 48 hours prior to inoculation and continuing throughout the course of the experiment.

Lesions of papular stomatitis similar to those in calves with naturally occurring cases appeared in all 3 calves on the 9th, 6th, and 11th days after inoculation, respectively. Calf B427 had lesions on the margins of the nostrils, hard palate, and gingiva which were still present on the 15th day when the calf died from enterotoxemia. Only one macroscopic lesion was detected in calf B428; it was present on the inner surface of the lower lip from the 6th to the 11th days after inoculation. When euthanasia was performed on the 28th day, however, microscopic lesions of papular stomatitis were found on the margins of the nostrils, lumbar skin, reticulum, and omasum. The lesions in calf B429 were limited to the edges of the nostrils from the 11th to 18th day when euthanasia was performed.

Tissue Culture-Passaged Material.—Four Jersey calves (B1685, B1686, B1687, B1688) were used to test the pathogenicity of tissue culture-passaged materials. All 4 calves were from the same farm and were less than one month old.

Ten calves served as controls. One calf, B1687, was placed in isolation and inoculated with nutrient medium from parallel, serially passaged but uninfected tissue cultures. The 9 remaining calves served as uninoculated controls and were housed on the farm from which the healthy experimental calves were obtained. Nutrient medium from the 5th serial passage in bovine kidney tissue cultures of a suspension of lip from calf B426 served as the inoculum. Each calf was inoculated with 0.03 ml. within the mucosa of the lower lip, 0.10 ml. beneath the mucosa of the lower lip, 0.20 ml. on the scarified mucosa of the lower lip, 0.20 ml. on the scarified ventral surface of the tongue, 0.20 ml. on the scarified margin of one nostril, 0.50 ml. intradermally in 2 sites on the neck, and 2.0 ml. intramuscularly. Calves B1685 and B1686 were given nutrient medium representing a dilution of at least 1×10^8 of the original tissue suspension. Calf B1688 was inoculated with nutrient medium that had been forced through a Seitz ST-1 filter pad under 2 lb./sq. inch positive pressure.

Thirty-six hours after inoculation, a typical lesion of papular stomatitis developed at the scarification site in the lip of calf

B1685. Secondary lesions appeared the 4th day on the mucosa of the lower lip and margin of one nostril and later on the muzzle and margin of the other nostril. Although there was regression of the primary lesion on the 3rd day, the others slowly enlarged. By the 14th day, the floor of one nostril was covered with a papillomatous plaque consisting of numerous, nearly confluent papules. Euthanasia was performed the 16th day after inoculation and additional microscopic lesions of papular stomatitis were found in a buccal papilla and on a pillar of the rumen. No lesions were detected at the intra- and sub-mucosal injection sites on the lip or in the skin or muscle.

Papular lesions appeared at the inoculation site on the floor of the nostril of calf B1686 on the 3rd day after inoculation. On the 5th day, a small lesion was present 5.0 mm. away. On the 10th day, lesions appeared in the other nostril, and on the 27th day on the mucosa of the lower lip and dental pad. The latter regressed in a few days but the lesions on the margins of the nostrils slowly increased in size. On the 40th day, a swab from the lesion in the nostril was rubbed into an abraded area on the inner surface of the lower lip. A typical proliferative lesion appeared 2 days later. On the 49th day after the 1st inoculation, all the lesions disappeared except the one on the lip, which was still present when euthanasia was performed on the 92nd day. Additional focal microscopic lesions were found in the omasum.

A lesion of papular stomatitis appeared at the scarification site on the lip of calf B1688 on the day after inoculation. On the 8th day, lesions appeared at the inoculation site in the margin of the nostril, and lesions persisted on both sites until the 13th day, when the calf died from enterotoxemia. One of the 10 controls, calf B1687, died on the day after inoculation from an interventricular septal defect. On the 20th day after inoculation, however, the 9 control calves on the same farm from which the experimental animals had been obtained (some of them in the same pen) were examined and no evidence of papular stomatitis was detected.

Pathogenicity for Laboratory Animals and Tissue Cultures.—Three series of experiments were performed to determine the pathogenicity of the virus for laboratory animals and tissue cultures. The inoculums for the 1st series of experiments were suspensions of brain, lungs, spleen, and thymus from calf B137, and brain, lungs, and spleen from calf B167. Some of these materials had been inoculated into calves (Table 1). Embryonating eggs were inoculated via yolk sac, amniotic cavity, and chorioallantoic membrane routes. As many as 7 serial passages at 6-day intervals were made by the latter route. A pool of chorioallantoic membranes from one of these series was inoculated into calves B427, B428, and B429. There were no deaths of embryos, disturbances of growth, or macroscopic lesions in more than 200 embryonating eggs. Randomly selected embryos were examined microscopically, but no lesions were found. Antibiotics were not routinely used; no microorganisms were found in smears stained by the Macchiavello method.

Three-week-old mice were inoculated intranasally. After the 2nd passage, no macroscopic or microscopic lesions were seen. Several groups of bovine kidney and feline kidney tissue cultures were inoculated with no cytopathic effects detected.

The 2nd series of experiments originated with a suspension of lip lesions from naturally infected calf B426. Three serial passages at 5-day intervals were made via the yolk sac in embryonating eggs, and 3-week-old mice were inoculated intranasally, intraperitoneally, and intracerebrally. There was no evidence of disease.

In the 3rd series of experiments, 5 serial passages in bovine kidney tissue cultures were made at 5-day intervals with each of the 10 different materials suspected of containing the virus of papular stomatitis. These materials included fresh swabs of lesions, triturated suspensions of lesions from both naturally occurring and experimentally produced cases, and whole blood. No cytopathic effects or inclusion bodies were detected. Virus was demonstrated by inoculation of calves (Table 1) with nutrient medium from the 5th passage of one series that started with a suspension of lip from calf B426. Using portions of this known infective material, 3 serial passages each were made in embryonating eggs by the yolk sac, amniotic cavity, and chorioallantoic membrane routes, 2 passages were made in 3-week-old mice by the intracerebral and intraperitoneal routes, and 3 passages were made at 5-day intervals in 1-day-old mice by the intracerebral and intraperitoneal routes.

There was no evidence of disease clini-

cally or microscopically at necropsy. No disease developed during 2 weeks in guinea pigs inoculated intradermally in the foot pads. Tissue cultures inoculated with the known infective tissue culture fluid included: kidney and lungs of newborn calf, porcine kidneys, HeLa cells, monkey kidneys, and guinea pig kidney cells. All these cells were maintained in nutrient medium containing plasma which had been collected from a newborn calf that had never nursed. No cytopathic effects or inclusion bodies were observed.

DISCUSSION

In Europe, the first attempts to transmit papular stomatitis came about accidentally. About 1900, papular stomatitis was confused with foot-and-mouth disease. It was the practice at that time, when one animal became sick with foot-and-mouth disease, to expose the entire herd by oral transfer of saliva so they would all become immune. Papular stomatitis was not reproduced in this way.[3, 5, 13]

Since then, papular stomatitis has been reproduced in calves by the intramucosal injection or scarification in the mouth with portions of lesions[4, 10, 15, 16] and by subcutaneous or intravenous injection with blood.[10] On one occasion, Ostertag and Bugge [10] were successful with the inoculation of filtered serum. Schaaf *et al.*[16] also detected the virus in the blood but were able to demonstrate it in only 1 of 4 cattle. The same authors indicated that transmission occasionally resulted from restraint by the caretakers since typical lesions were found around the nares. In many of our cattle with naturally occurring cases,[1] however, we found primary lesions in the margins of the nostrils of calves that had not been restrained by the nose.

Ostertag and Bugge [10] found no evidence of disease when portions of oral lesions were artificially transmitted to the nasal mucosa, conjunctiva, or vaginal mucosa. Schaaf *et al.*[16] were unable to transmit the disease by scarification of teats, udder, penis, scrotum, or shaved skin of the neck and abdomen. Plowright and Ferris [12] were unsuccessful with intradermal inoculation of the neck or inoculation in the conjunctival sac. Our results were identical; no lesions developed at the intradermal inoculation sites in the neck, at the intramuscular injection sites, or in the conjunctival sacs in 3 of our calves. The disease was readily reproduced, however, by introduction of viral suspensions or swabs from lesions into the scarified oral mucosa. Scarification of the oral mucosa

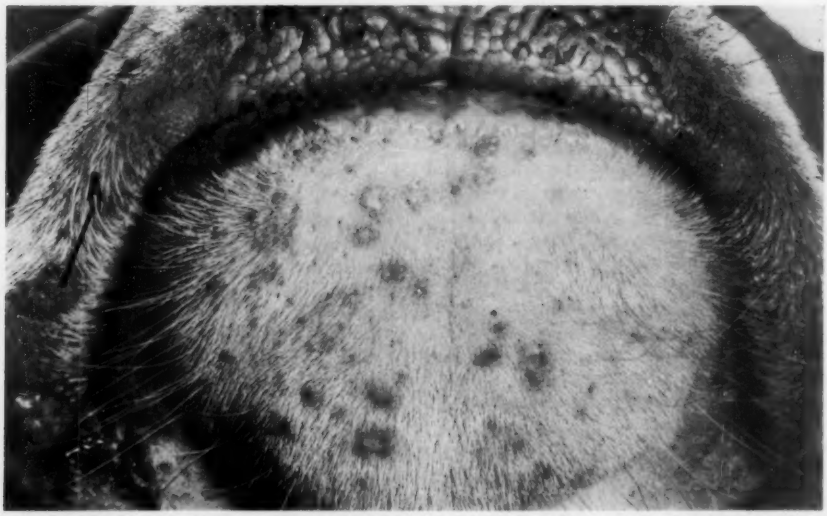

Fig. 3—Circular lesions on the chin of calf B777. Each lesion had a raised, roughened, yellowish brown center and a bright red peripheral ring. A papular nodule (A) can be seen on the margin of the lip.

Fig. 4—Large circular lesion (arrows) on the muzzle of calf B778, 20 days after inoculation. The lesion was gray and slightly convex in the center, with a peripheral yellowish brown ring. The normally black muzzle had large areas of gray discoloration indicating the sites of previous lesions.

without inoculation did not cause lesions in 20 control calves.

The incubation periods of calves inoculated with egg-passaged material were a few days longer than expected (Table 2); this might have been a reflection of attenuation or decrease of titer of the virus in the chicken embryo passages. Schaaf *et al.*[16] were unsuccessful in an attempt to produce the disease with egg-passaged material.

Judging from the results presented in Table 2, there was no loss of pathogenicity of the virus propagated in tissue culture. In spite of a dilution of at least 1×10^8 of the original tissue suspension and 5 passages in bovine kidney tissue cultures, the pathogenicity was retained when tested in calves. Seitz-filtered as well as nonfiltered tissue culture-passaged virus retained its pathogenicity and produced infection after an incubation perod of 2 to 3 days in comparison with an incubation period of 3 to 5 days in calves with the original suspensions of lip lesions from naturally infected animals. Plowright and Ferris[12] in East Africa also reproduced the disease in calves with virus isolated in tissue culture.

Only Ostertag and Bugge,[10] Schaaf *et al.*,[16] and Plowright and Ferris[12] gave a description of the experimentally produced disease. Our cases were identical with theirs and with the naturally occurring disease previously described.[1] Systematic study of all organs at necropsy confirmed the striking restriction of lesions to the areas in and around the mouth. Pretreatment with steroids had no apparent effect on the course of the disease in 3 animals.

Since completion of our tissue culture studies, Plowright and Ferris[11] have demonstrated a cytopathic effect and inclusion body formation in cultures of bovine testes. Various bovine isolates may vary considerably in their ability to destroy tissue culture cells. In view of the proliferative rather than destructive effect of the virus on epithelial cells *in vivo*, we were not surprised to find multiplication without morphologic evidence in our tissue cultures.

On the basis of our work and the experiments referred to, it is concluded that papular stomatitis can be readily reproduced by scarification of the oral mucosa or parenteral inoculation of calves with portions of lesions, the virus isolated in tissue cultures, or on some occasions with blood from an affected animal. The distribution of lesions in experimentally infected calves is the same following either local or parenteral inoculation. No attempts have yet been

made to demonstrate the virus in feces, although it is undoubtedly present there and could account for some of the recent reports of bovine enteric orphan viruses.[6,8]

Our attempts to infect mice, guinea pigs, and chicken embryos with the virus of papular stomatitis were unsuccessful. As yet, cattle have been the only animals found susceptible to papular stomatitis. Attempts have been made to infect horses, sheep, goats, pigs, dogs, rabbits, suckling and weanling mice, and embryonating eggs.[9,14,16] Except for the experiment of Olson and Paliones [9] with a disease similar to papular stomatitis, in which 1 of 6 dogs developed papillomatous lesions at the inoculation site, all have been unsuccessful.

The recent discovery of cytopathogenic strains of virus and the characterization of the disease which enables the virus to be identified should stimulate studies on the nature of the virus and its relationship to other viruses. Schaaf *et al.*[16] were able to store the virus in glycerin at 2 C. for at least 5 months. We found suspensions of lesions still infective after 76 days at −65 C.

SUMMARY

Bovine papular stomatitis was produced in 10 of 15 experimental calves; 32 controls remained free of infection. The virus of bovine papular stomatitis was successfully transmitted to 3 calves by tissue culture material, to 3 calves by chicken embryo chorioallantoic membranes, to 1 calf by a swab from a lip lesion, to 2 calves by suspensions of lip and palate lesions, and to 1 calf placed in a pen with a naturally infected calf. Five calves exposed to suspensions of internal organs did not contract the infection.

The experimentally induced disease was identical to the naturally occurring infections from which the virus was obtained. Proliferative, papular lesions developed at inoculation sites in the mouth or nostrils and in a few days secondary lesions appeared in the mouth, on the margins of the nostrils, on the muzzle and skin around the mouth, and sometimes in the esophagus and 1st 3 compartments of the stomach. Lesions were present as long as 98 days after experimental exposure. No cytopathic effects were detected in tissue cultures of bovine kidneys, bovine lungs, porcine kidneys, feline kidneys, monkey kidneys, HeLa, or guinea pig kidney cells. Nutrient medium from the 5th passage in bovine kidney cells, however, produced the disease when inoculated into calves. Guinea pigs, weanling and suckling mice, and embryonating eggs were not susceptible to the virus of bovine papular stomatitis.

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SUMMARIO IN INTERLINGUA

Bovin Stomatitis Papular. II. Le Morbo de Production Experimental

Bovin stomatitis papular esseva producite in 10 ex 15 vitellos experimental. Un gruppo de controlo de 32 vitellos remaneva libere de infection. Le virus de bovin stomatitis papular

esseva transmittite a bon successo a 3 vitellos per material de histoculturation, a 3 vitellos per membranas chorioallontoic de embryones de gallina, a 1 vitello per un tampon ab un lesion labial, a 2 vitellos per suspensiones ab lesiones labial e palatal, e a 1 vitello per placiar lo in un stand insimul con un naturalmente inficite vitello. Cinque vitellos exponite a suspensiones de organos interne non contraheva le infection.

Le experimentalmente inducite morbo esseva identic con le naturalmente occurrente infectiones ab que le virus esseva obtenite. Proliferative lesiones papular se disveloppava al sitos del inoculation in le bucca o nares, e intra alicun dies lesiones secundari appareva in le bucca, al margines del nares, e in le musello e le pelle circum le bucca, a vices etiam in le esophago e le prime 3 compartimentos del stomacho. Lesiones esseva presente durante periodos de usque a 98 dies post le exposition experimental. Nulle effectos cytopathic esseva detegite in histoculturas de renes bovin, le pulmones bovin, de renes porcin, de renes felin, de renes simian, le cellulas HeLa, o de cellulas renal de porco de India. Tamen, le medio de nutriente ab le quinte passage in cellulas de ren bovin produceva le morbo quando illo esseva inoculate in vitellos. Porcos de India, muses de etate atettate o dislactate e ovos embryonante non esseva susceptibile pro le virus de bovin stomatitis papular.

Bovine Papular Stomatitis. III. Histopathology

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HISTOPATHOLOGIC STUDIES of bovine papular stomatitis have in previous reports given consideration to few organs other than those containing grossly visible lesions.[3, 4, 6] In view of the hematogenous spread of the virus, widely disseminated lesions might be expected. The purposes of this study were to survey all the major tissues of affected calves microscopically to determine the distribution of lesions and to characterize the microscopic appearance of the lesions as an aid in differential diagnosis.

This report is based on 6 naturally occurring and 10 experimentally produced cases of papular stomatitis. The clinical and necropsy findings, as well as isolation of the virus and reproduction of the disease, have been previously described.[1, 2]

MATERIALS AND METHODS

After necropsy, the following tissues were examined microscopically: lumbar skin, muzzle, lip, tongue, hard palate, soft palate, tonsils, nostrils, turbinate, trachea, bronchial lymph nodes, each lobe of the lung, heart, aorta, iliac lymph nodes, mandibular lymph nodes, parotid salivary gland, esophagus, rumen, reticulum, omasum, abomasum, duodenum, pancreas, jejunum, ileum, cecum, colon, mesenteric lymph nodes, liver, gallbladder, kidneys, urinary bladder, mammary gland, testes or ovaries, seminal vesicle or uterus, thyroid glands, adrenal glands, thymus, pituitary gland, spleen, rib at the costochondral junction, bone marrow, psoas muscle, diaphragm, eyelids, eyes, cerebrum, thalamus, midbrain, pons, cerebellum, medulla, cervical spinal cord, thoracic spinal cord, lumbar spinal cord, and all macroscopic lesions. Tissues were fixed in 10% buffered isotonic formalin, embedded in paraffin sectioned at 6 μ, and stained with hematoxylin and eosin. Special stains and histochemical methods included Giemsa, Grocott, Gridley fungus, Wilder reticulum, and periodic acid-Schiff.

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RESULTS

The distribution of lesions in the naturally occurring and experimentally induced disease was similar and is presented (Table 1). Only positive findings are presented; the organs where no histopathologic changes were demonstrated are not listed. The earliest changes detected microscopically were focal hydropic degeneration and epithelial hyperplasia of the mucosa and epidermis (Fig. 1). The mucosa was thickened in nearly every instance and was often twice normal thickness, with long, slender rete pegs. The foci of hydropic degeneration were about 200 to 300 μ in diameter and located in the superficial stratum spinosum (Fig. 2). Under low magnification, they resembled spongy areas of ballooning degeneration, but closer examination revealed the cells to be only slightly enlarged, normal, or even decreased in size. The nuclei were greatly shrunken

Fig. 1—Focal hydropic degeneration and thickening of the mucosa—hard palate of calf B427. H & E stain; x 85.

and distorted and sometimes fragmented. The cytoplasm was empty-appearing except for a network of very fine fibrils and granules. The latter were strongly positive with the periodic acid-Schiff reaction so that early lesions, which might have been easily overlooked upon casual examination, were readily demonstrated (Fig. 3). Macroscopically, such lesions appeared as scarcely discernible, elevated, white spots.

As the disease progressed, the foci of degeneration moved closer to the surface by the normal proliferation of basilar epithelium. Within the cytoplasm and occasionally in the nucleus were homogenous, eosinophilic, discrete, circular inclusion bodies from 2 to 10 μ or more in diameter (Fig. 4). There was usually only one in a cell; they could not be demonstrated in every case. Intercellular bridges disappeared. Between the cells were tiny vacuoles containing nuclear debris. In the subjacent papillary lamina propria, there was distention of capillaries with blood, a mild increase in histiocytes and, infrequently, infiltration of a few lymphoid cells. Such lesions appeared macroscopically as circular pink spots or as small papules with narrow red margins, according to the amount of mucosal thickening. The focal nature of the lesions was apparent microscopically in even the largest confluent lesions.

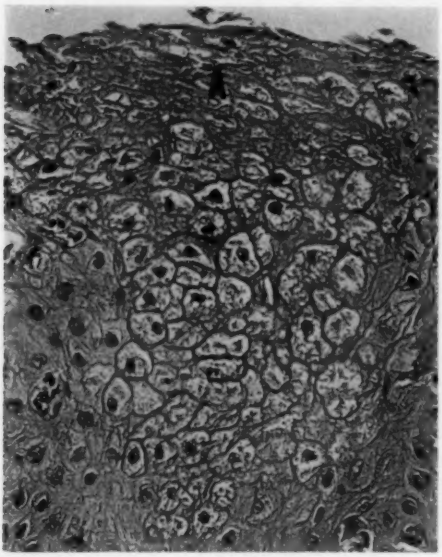

Fig. 2—Higher magnification of the hydropic degeneration in Figure 1. Notice the disruption of the stratum corneum (A). H & E stain; x 310.

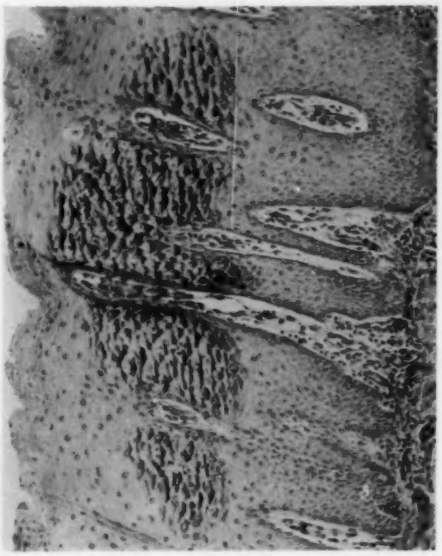

Fig. 3—A focus of papular stomatitis in the lip of calf B779, sharply delineated by the presence of PAS-positive granules in the affected cells. Periodic acid-Schiff reaction; x 113.

When the degenerative process neared the surface, the layers of the stratum corneum were disrupted and, grossly, the lesions appeared slightly roughened. Bacteria were found only in these disorganized surface layers. Many of the epithelial cells had collapsed and taken the form of nests of globular, acellular, eosinophilic masses up to 50 μ in size, containing small amounts of nuclear debris (Fig. 5). The presence of granular epithelial cells and the formation of eosinophilic globules suggested abortive keratin formation. At this stage, there was no longer subjacent congestion, and the deeper layers of the stratum spinosum appeared normal. Grossly, these lesions had a reddish or yellowish brown color that did not blanch on digital pressure and persisted for many days, presumably until healing took place as the cells were replaced from below.

In nearly half the lesions studied, most often in the margins of the nostrils and in the rumen, reticulum, and omasum, there

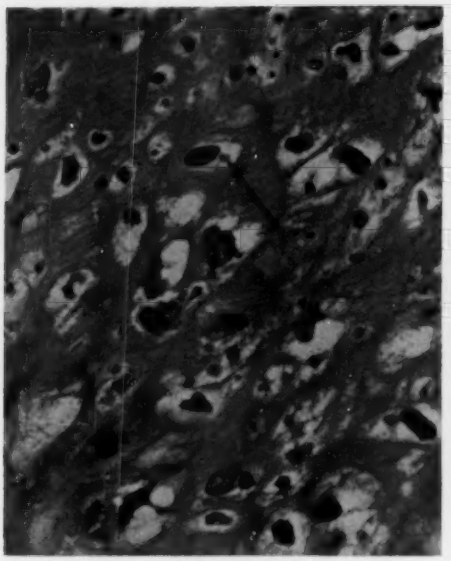

Fig. 4—Inclusion bodies (arrow) in a more advanced focus of papular stomatitis; also pyknosis and karyorrhexis. Lower lip of calf B426. H & E stain; x 735.

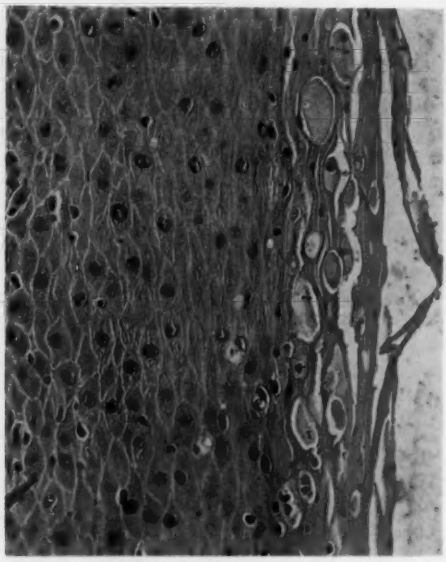

Fig. 5—A nearly healed lesion in the lip of calf B1344. Acellular, amorphous globules and a few neutrophils in the stratum corneum. H & E stain; x 270.

was infiltration of the degenerative focus by neutrophils (Fig. 6). This was not associated with the presence of bacteria. Occasionally, the basilar epithelium was disrupted by migrating neutrophils. As the lesions progressed, neutrophils persisted in the degenerative foci, and when the lesions were nearly healed, neutrophils were found in small clusters in the stratum corneum.

As indicated (Table 1), the esophagus, rumen, reticulum, and omasum were sites of papular lesions. The most severe were in calf B617 in which the lesions took the form of papillary nodules measuring 1 to 4 mm. in diameter, and elevated 1.5 mm. above the esophageal mucosa. The nodules were brown to red in color and consisted of hyperplasia of the stratum spinosum with superficial erosion and ulceration (Fig. 7).

TABLE 1—Distribution of Lesions in Papular Stomatitis

Calf No.	Nostril	Lip	Muzzle	Palate	Skin	Dental pad	Dental plate	Buccal papillae	Omasum	Rumen	Reticulum	Esophagus	Tongue
B 167	..	..	..	..	..	..	..	..	+	..	..	..	..
B 617	..	..	..	..	..	..	..	..	..	+	..	+	..
B1344	+	+	+	+	+	+	+	+	..	+	+	+	..
B1345	+	+	+	+	..	+	+	+	..	..	..	..	..
B 425	+	..	..	..	..	..	..	..	..	..	..	..	..
B 426	+	+	+	+	+	+	+	+	..	..	..	..	..
B 638	+	+	+	+	+	..	+	..	+	..	..	..	..
B 777	+	+	+	+	+	+	+	..	..	..	..	..	+
B 779	..	+	+	+	..	..	..	+	+	..	+	..	+
B 778	+	+	+	+	+	+	+	+	..	..	..	..	..
B 427	+	+	..	+	..	..	..	..	..	..	..	..	..
B 428	+	+	..	..	+	..	..	..	+	..	..	..	..
B 429	+	..	..	..	..	..	.	..	+	..	+	..	..
B1685	+	+	+	..	..	..	..	+	..	+	..	..	..
B1686	+	+	..	..	..	+	..	..	+	..	..	..	..
B1688	+	+	..	..	..	..	..	..	..	..	..	..	..
Total	13	12	8	8	6	6	6	6	6	3	3	2	2

In the mucosa at the margins of the ulcerations were small foci of hydropic degeneration similar to the oral and lip lesions seen in the majority of calves studied. The lamina propria within and at the base of the nodules was hyperemic and edematous.

In calf B1344, lesions were distributed throughout the length of the esophagus. On the mucosal ridges, there were superficial, oval, reddish brown erosions 1 to 3 mm. in diameter and raised, linear, roughened streaks up to 1.0 cm. long. Near the cardia was an elliptical ulcer measuring 7 mm. long, 2 mm. wide, and 1.0 mm. deep. Microscopically, the lesions were abrupt, steep-walled erosions and ulcerations. The mucosal defects were filled with neutrophils, fibrin, cellular debris, and bacterial colonies, but the inflammatory response was limited to a zone of granulation tissue 1.0 mm. in diameter at the base of each lesion. In the mucosa at the margins of the ulcerations was mild pseudoepitheliomatous hyperplasia.

Among the 16 calves, proliferative lesions and microabscesses were found in the rumen of 3, the reticulum of 3, and the omasum of 6 animals.

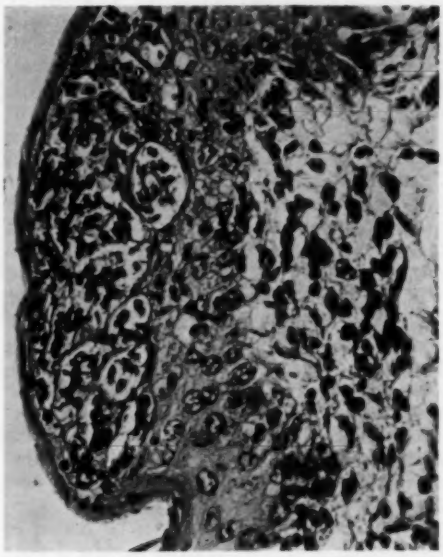

Fig. 6—An intramucosal focus of hydropic degeneration and infiltration of neutrophils in the omasum of calf B428. H & E stain; x 375.

Discussion

The reaction of the tissues to the virus was primarily proliferative with some degenerative changes. The excessive proliferation expected with papillomatosis, the necrotic changes associated with the mucosal diseases and rinderpest, and the severe ballooning and intercellular edema associated with the vesicular diseases are unlike papular stomatitis. Proliferation and hydropic degeneration are common responses to many viral infections in the skin and mucosae of ruminants. This is probably not evidence that the viruses are related but rather that stratified squamous epithelial tissues are limited in their possible responses to invasion by a virus.

Extensive microscopic study of all the organs in the body revealed very few lesions in locations other than those observed at necropsy. Lesions have been reported previously in the esophagus in 2 cattle by Ostertag and Bugge [3] and in 2 others by Pallaske.[4] In addition, Pallaske found one papule in the rumen that was modified by fungi. Lesions have not previously been described in the reticulum and omasum. We found lesions in the esophagus or first

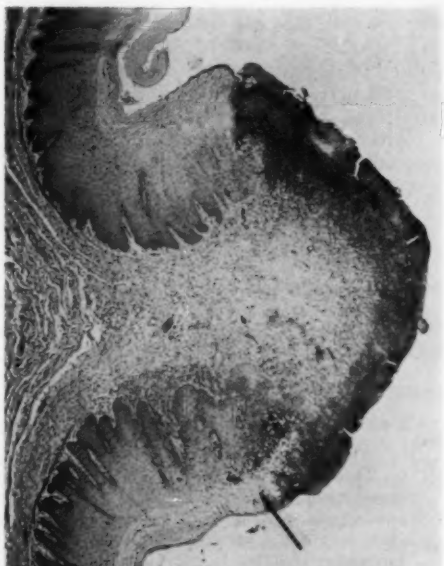

Fig. 7—Photomicrograph of a large nodule in the mucosa of the esophagus of calf B617. Notice the great elevation of the mucosa, the superficial ulcer and the focal hydropic degeneration (arrow) at the margin of the ulcer. H & E stain; x 21.

3 compartments of the stomach in 9 of the 16 calves studied (Table 1). The incidence might have been even higher had they all been destroyed at the same stage of the disease. The esophageal lesions in one of the cases of Ostertag and Bugge [3] and in calf B1344 were elliptical erosions and ulcers rather than papules, suggesting the healing stages after sloughing of papules and invasion of bacteria. The fact that lesions had been present for 54 days in other parts of the body and had healed was further reason to believe that the esophageal lesions seen at necropsy in calf B1344 represented advanced and healing processes. Before this study we had observed lesions of papular stomatitis in the reticulum and omasum of calves and erroneously attributed them to normal retrogressive changes. The incidence of this type of lesion is high in our routine necropsy material.

Skin lesions were found in 2 cattle in the lumbar area, but it is not known if they were caused by the papular stomatitis virus. Ostertag and Bugge [3] noted dirty gray-brown crusts in the skin of some of their experimental calves. It is possible that lesions occur in the stomach and intestines but that the nonstratified epithelium is quickly repaired. In 2 herds near Columbus, Ohio, naturally occurring papular stomatitis was associated with mild, intermittent diarrhea and anorexia.

Unlike Pallaske,[4] we were unable to demonstrate inclusion bodies in every case; they may not be present at every stage of the disease. Many of the larger inclusions had a striking tinctorial resemblance to keratin and may represent abortive keratin formation (Fig. 5). Reczko [5] demonstrated bodies resembling viral particles in inclusion material under the electron microscope.

SUMMARY

All major organs from 6 cattle with naturally occurring and 10 with experimentally produced cases of papular stomatitis were studied histopathologically. Lesions were found in the esophagus and first 3 compartments of the stomach as well as in and around the mouth. The principal microscopic changes were focal hydropic degeneration, hyperplasia of the mucosa or epidermis, and the formation of intracytoplasmic inclusion bodies.

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SUMMARIO IN INTERLINGUA

Bovin Stomatitis Papular. III. Histopathologia

Omne le major organos de 6 pecias de bestial con naturalmente occurrente stomatitis papular e de 10 pecias de bestial con stomatitis papular de production experimental esseva studiate histopathologicamente. Lesiones esseva trovate in le esophago e le prime 3 compartimentos del stomacho e etiam in e circum le bucca. Le major alterationes microscopic esseva focal degeneration hydropic, hyperplasia del mucosa o del epiderme, e le formation de corpores de inclusion intracytoplasmatic.

Studies on Bovine Portal Blood. II. Blood Flow Determinations with Observations on Hemodilution in the Portal Vein

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MEASUREMENT of portal blood flow in ruminants has been of particular interest in recent years because of its possible use in conjunction with portal analysis for the quantitative estimation of the end-products of digestion. From the anatomical arrangement of the veins forming the portal system in sheep, and from the results of various *in vivo* and *in vitro* tests, Shambye[8] concluded that the indicator dilution method could be used for the measurement of portal blood flow. Shambye's 2nd publication[9] provides the only information available on the portal blood flow in unanesthetized ruminants.

In the course of the portal blood flow studies, it was observed that the blood cell volume of the portal vein blood was lower than that of arterial blood. Blackwood and Sterling[3] reported that in cattle jugular vein hematocrit values and hemoglobin levels were consistently higher than those of the carotid artery. They attributed the differences to hemoconcentration resulting from salivary secretion. If secretion of saliva is sufficient to produce a detectable difference in concentration between the carotid artery and the jugular vein, it is possible that the hemodilution between the arterial and portal systems could occur as a result of water absorption or some other physiologic process. However, Annison *et al.*[1] reported that there was good agreement between the hemoglobin content of portal and arterial blood.

Results of portal blood flow determinations using the indicator dilution method in unanesthetized cattle are presented in this report. Because of the possible importance of hemodilution in difference studies, the results of some of our observations are also included.

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From the departments of Dairy (Fries) and Surgery and Medicine (Conner), Michigan State University, East Lansing. Published with the approval of the director of the Michigan Agricultural Experiment Station as Journal Article No. 2520.

Dr. Fries is now with the Dairy Cattle Research Branch, USDA, Montana State College, Bozeman.

MATERIALS AND METHODS

Blood Flow.—The portal blood flow determinations were from 4 Holstein-Friesian calves, weighing from 112 to 239 kg. Polyethylene catheters were established in the portal vein at the porta hepatis and in a branch of the mesenteric vein. The details of the surgical procedures and the maintenance of the catheters have been presented in a previous paper.[5] The location of the catheters in each animal was confirmed by necropsy at the termination of the study.

The use of the indicator method in determining blood flow has been reviewed by Dow.[6] The specific methods used in this study are essentially the same as those of Shambye.[9] A 2% solution of Evan's blue dye in 0.85% NaCl solution was used as the indicator in these studies. An accurately measured amount, either 2 or 3 ml. of the solution, was injected into the catheter in the mesenteric vein. The injection was followed instantaneously by flushing with 15 ml. of 0.85% saline solution to assure that all of the dye in the catheter reached the portal vein. Blood samples were collected from the portal vein at 2-second intervals for the 1st 40 seconds after injection by means of an automatic syringe. The Evan's blue dye concentration in the samples was determined, and resulting data were used for the estimation of portal blood flow, using the methods described by Shambye.[9]

Because of the preliminary nature of the studies, no attempt was made to maintain a uniform relationship between the time of flow determination and the time of feeding or other management practices. The 2 determinations on the day of surgery were made immediately on completion of the operation, while the animal was still showing the effects of anesthesia. In cases where 2 determinations were made on the same day, the 2nd determination followed the 1st by a period of 15 minutes, considered adequate for uniform dispersion of the indicator throughout the general circulation.

Packed Cell Volume.—The observations made on differences in packed cell volumes (PCV) were from 2 calves used in the blood flow studies. An exteriorized carotid artery had been established in calf 13. A number of comparisons of PCV of the carotid artery and portal vein blood were made at irregular intervals or in conjunction with other studies. The portal blood samples were obtained without restraint or visible disturbance to the animal. Considerable excitement was evoked when obtaining carotid samples. Samples from the 2 sites were obtained with as short an inter-

vening time interval as possible, usually less than 1 minute. Sampling from the portal vein preceded that from the carotid artery.

An exteriorized carotid artery had not been established in calf 15. A number of comparisons between the jugular and portal veins were made, following the same procedures as for the preceding animal. Excitement did not appear to be a problem when obtaining the jugular samples.

Because of the possibility of excitement confusing the results of the early observations, a small study was conducted with calf 15, in which the jugular and portal samples were obtained simultaneously. Immediately following this, the calf was given 5 ml. of 1:1,000 epinephrine hydrochloride (i.v.) or 60 mg. of pilocarpine hydrochloride (i.m.). A 2nd set of simultaneous samples was obtained as soon as the calf developed clinical effects of the drugs. Control experiments in which no drug was administered were conducted with the 2nd set of samples collected 10 minutes after the 1st set. Each experiment was duplicated and only 1 experiment was conducted on a given day.

The PCV determinations were made by means of Wintrobe tubes, centrifuged at 3,000 r.p.m. for 30 minutes. All determinations directly compared were run simultaneously in order to eliminate any variations between samples.

RESULTS AND DISCUSSION

Blood Flow.—The results of the portal blood flow determinations are summarized (Table 1). Because of difficulties encountered in maintaining the catheters, the number of determinations was limited. However, several preliminary conclusions can be made concerning the magnitude of blood flow and some of the causes of its variation.

TABLE 1—The Rate of Blood Flow Through the Portal Veins of Calves

Determination No.	Calf No.	Weight	Time after surgery	Portal blood flow	
		(kg.)	(days)	(ml./min.)	(ml./min./kg.)
1	10	135**	4	8,940	65.7
2*	12	112	0	1,460	12.0
3			3	6,850	56.2
4			4	6,730	55.2
5			6	2,210	18.1
6†			6	1,960	16.1
7			7	4,670	38.2
8	13	181	2	3,680	20.3
9			6	5,170	28.4
10			7	11,130	61.3
11†			7	2,610	14.4
12*	15	239**	0	7,170	30.0
13			1	5,600	23.4
14†	15	187	1	1,730	9.2
15†			1	2,830	15.1

* Measurements made immediately following surgery.

** Estimated weight.

† The determinations followed the preceding determinations by a period of 15 minutes. Determination No. 11 followed the deliberate provocation of the animal to an excited state.

Except when 1 animal was deliberately provoked (determination 11, Table 1), the animals were not visibly excited or otherwise disturbed when the collections were made, and no restraint was required. The values obtained at 15-minute intervals (determinations 5–6 and 14–15) had approximately the same magnitude. The differences were not large when the range of all values was considered, indicating that the portal blood flow is relatively stable during short time intervals and that the method is fairly repeatable.

Exciting the animal resulted in a decrease of portal blood flow from 61.3 to 14.4 ml./min./kg. (determinations 10 and 11). A decrease would be expected because of the marked vasoconstriction which occurred in the visceral regions when the animal was excited. Fegler and Hill[7] found a close relationship between portal blood flow and cardiac output in anesthetized sheep. They suggested that if the relationship held in conscious animals, a good approximation of portal blood flow could be made by determining cardiac output. If this relationship holds in the conscious animal, it is probably only under a limited set of conditions. In conditions such as excitement, one would expect a considerable increase in cardiac output with a decrease in portal flow. It is not inconceivable that under some conditions a reverse situation may occur.

The determination of portal blood flow made immediately following surgery, while the animal was showing the effects of general anesthesia (determination 2), was considerably lower than the average of the other determinations. It has been shown that portal blood flow measured in the acute experiments* was lower than that measured in the chronic experiments, and that pentobarbital sodium will depress flow in chronic experiments.[9] The determination (12) made under local anesthesia was lower than the average, but not to as great a degree as during general anesthesia.

The average of the determinations, excluding those made under abnormal condi-

* Acute experiments refer to those in which efforts are not made to save the life of an animal. Data are collected during or shortly after surgery but while there are still effects of the surgical anesthesia. In chronic experiments, the animals are allowed to recover from the immediate effects of surgery and anesthesia before data are collected; they are permitted to live as nearly normal as possible.

tions (2, 11, and 12), was 37.8 ml./min./kg. This is in good agreement with the average of 37.0 ml./min./kg. obtained from conscious sheep by Shambye.[9] The values obtained during the first 2 days after surgery, while within the range of the other determinations, were considerably lower than the general average. The animals were usually depressed at this time and were not eating normally, which may have been a cause of the low portal blood flow values that were obtained. If these values are excluded, the average flow was 46.0 ml/min./kg. This figure compares closely with the average of 45.8 ml./min./kg. that Fegler and Hill predicted for the conscious sheep during acute experiments.

The close agreement between these results in calves and those in sheep suggests that the dynamics of portal blood flow are similar in both species. This would allow fairly general application of results from one species to the other. The normal range of portal blood flow in the resting calf appeared to be 20 to 60 ml./min./kg. of body weight, with an average of approximately 40 ml./min./kg. However, since most of the experimental procedures disturb the animal, it is possible that the normal rate of flow would be somewhat higher. Considerably more data than is currently available would be required to determine if this is the case.

In order to use portal blood flow data in conjunction with difference data for absorption studies, the nature and causes for the variation must be characterized and eliminated or minimized in those cases when it is obviously not a function in the feeding regimen. Because of the variations in portal blood flow, a figure representing the average flow may give misleading results if used with difference data to determine absorption rate of a certain substance. The use of portal blood flow data from acute experiments may also be erroneous because of the depressed flow under these conditions.

Packed Cell Volume Differences.—Ten comparisons between the portal and carotid PCV values were made with calf 13. In all cases, the PCV was higher in the carotid artery than in the portal vein, with averages of 37.1 and 34.0% for the 2 sites, respectively. Similarly, with 12 determinations on calf 15, the PCV averages were 24.8 and 23.6% for the portal and jugular veins, respectively. In both cases, the differences were highly significant ($P < 0.01$) when analyzed by the "Student's t" test.

Because of the influence of saliva production,[3] it could be expected that the difference would have been greater between the portal and jugular veins than between the portal vein and carotid arery. In addition to the animal difference, there are 2 factors which may partially account for the results obtained. Because of a severe hemorrhage resulting from an overdosage of dicoumarol, the PCV values for calf 15 were considerably lower than those for calf 13. On a relative basis, the difference is not so great.

The 2nd factor was the excitement evoked when blood samples were taken from the carotid artery of calf 13. Excitement causes splenic contraction with a rise in PCV.[2] Because of the time interval between samples, this may have been a factor in the difference observed between the 2 sampling sites.

In order to eliminate any effects of excitement, the study using simultaneous sampling was carried out on calf 15. The results are presented (Table 2). As before, the PCV was always higher in the jugular vein blood. In the control experiments, the values for the 1st and 2nd set of simultaneous samples were somewhat erratic but of small magnitude. When averaged, there was no significant variation in the PCV of blood from either vein or in the difference between them.

TABLE 2—Comparison of the Packed Cell Volume (%) of Simultaneous Portal and Jugular Vein Blood Samples and the Effect of Epinephrine and Pilocarpine on the Differences (Calf 15)

Date	Control values		Experimental values	
	Portal	Jugular	Portal	Jugular
CONTROL				
7–10–59	27.3	28.3	28.0	29.6
7–11–59	30.1	31.5	30.0	30.3
Average	28.7	29.9	29.0	30.0
EPINEPHRINE				
7–12–59	27.0	27.7	30.9	31.7
7–13–59	31.0	31.4	34.3	34.8
Average	29.0	29.6	32.6	33.3
PILOCARPINE				
7–14–59	31.0	32.2	33.4	36.2
7–15–59	28.0	29.4	32.0	34.8
Average	29.5	30.8	32.7	35.5

Epinephrine produced a uniform rise in PCV in blood from both veins, but did not change the difference between them.

The continued difference with the use of epinephrine indicates that excitement was not the sole factor causing the variations in the preceding observations. Under this treatment, there is practically no saliva production.[4] Therefore, the difference between blood in the jugular and portal veins under this condition could be expected to be the same as the difference between that in the carotid artery and portal vein.

When pilocarpine was used, there was a larger increase in PCV in blood from the jugular vein than in that from the portal vein, resulting in an increased difference between them. The calf was visibly drooling at the time of sampling. The increased difference can logically be attributed to increased water removal from the blood by greatly increased secretion of saliva.

Although there are not enough observations to allow a precise evaluation of the differences in the PCV of blood from the jugular and portal veins as influenced by epinephrine and pilocarpine injections, it is felt that data are sufficient to show the existence of differences. It is possible that excitement or other factors could cause PCV variations similar to those noticed following injection of the drugs. If these PCV variations resulting from excitement or other factors are ignored in collecting data for absorption studies, accurate conclusions cannot be reached.

SUMMARY

Four calves with polyethylene catheters in the portal vein were used to determine the rate of portal blood flow, using the indicator dilution technique. Evan's blue dye was used as the indicator. The average flow rate for 10 determinations in unanesthetized animals was 37.8 ml./min./kg., with the range from 12.0 to 65.7 ml./min./kg. Portal blood flow appeared to be considerably depressed for the 1st 2 days after establishment of catheters. If these values were excluded, the average was 46.0 ml./min./kg. Two determinations, made immediately following surgery and while the animal was under the effects of the anesthesia, were considerably lower than the values from the unanesthetized animal. Deliberately exciting the animal decreased flow from 61.3 to 14.4 ml./min./kg.

In a series of comparisons of the packed cell volume (PCV) of blood from the jugular vein, carotid artery, and the portal vein, the PCV was consistently lower in the portal vein, implying some type of dilution between the general circulation and the portal vein.

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SUMMARIO IN INTERLINGUA

Studios in Bovin Sanguine Portal. II. Determinationes del Fluxo de Sanguine con Observationes del Hemodilution in le Vena Portal

Quatro vitellos con catheteres de polyethyleno in le vena portal esseva usate in determinationes del fluxo de sanguine portal per medio del technica a dilution de indicatores. Blau de Evans esseva usate como indicator. Le fluxo medie, basate super 10 determinationes in non-anesthesiate animales, esseva 37,8 ml per minuta per kg, con valores minimo-maximal de 12,0 e 65,7 ml per minuta per kg. Le fluxo de sanguine portal pareva esser deprimite considerabile-

mente durante le prime duo dies post le establimento del catheteres. Quando iste deprimite valores esseva ignorate, le nivello medie esseva 46,0 ml per minuta per kg. Duo determinationes que esseva effectuate immediatemente post interventiones chirurgic quando le animales esseva ancora sub le effecto del anesthesia esseva considerabilemente plus basse que le valores trovate in non-anesthesiate animales. Post exeitar le animales intentionalmente, le fluxo declinava ab 61,3 ad 14,4 ml per minuta per kg.

In un serie de comparationes del volumines de cellulas paccate inter sanguines ab le vena jugular, le arteria carotic, e le vena portal, il esseva trovate que le valor pro le vena portal esseva uniformemente le plus basse. Isto signala le un o le altere typo de dilution inter le circulation general e illo in le vena portal.

A Pasture Trial Using 2 Immunizing Doses of a Parasitic Bronchitis Vaccine

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INFECTIVE LUNGWORM LARVAE, partially inactivated by irradiation, have been used to stimulate immunity to subsequent infection by normal larvae. Several experiments under a range of conditions have been carried out in an attempt to find the best method of vaccinating against parasitic bronchitis (husk) in the field.[1-5]

The efficacy of the technique was first shown by administering 1 fairly large dose of irradiated larvae (4,000) as the immunizing agent and challenging the immunity of these treated calves with a single sublethal experimental inoculation.[5,7] Two field trials using the single dose method were successful, although a smaller number (1,000) of larvae was used per calf. The first trial[5] consisted of 500 principals and 500 controls on commercial farms; the morbidity was reduced from 62% to 6%. The other trial[2] involved 12 controls and 15 principals on a heavily contaminated pasture; 3 principals and 10 controls died and there was a considerable difference in clinical signs and fecal larval outputs between treated and untreated animals.

It was obvious from these experiments that, if a single vaccinating dose was given a sufficient time before exposure to infection, there was a considerable amelioration of the morbidity and mortality, but that under conditions of heavy challenge protection was not complete. During the following winter, further experiments[3] showed that if the primary dose of vaccine was followed by another dose given 6 weeks later, complete protection against a heavy challenge resulted. In this experiment, not 1 of 30 principals developed any clinical, pathologic, or parasitologic signs of infection; all of the controls were severely affected.

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An opportunity arose for testing the 2-dose procedure under field conditions when a heavily contaminated pasture became available. In the late summer and autumn of 1958, 38 of 40 calves on this field had died of parasitic bronchitis. Such high mortality is seldom seen under farm conditions, and it was thought that this pasture might provide a rigorous challenge to the vaccine under natural conditions of ingestion of larvae. Despite the fact that it was rather late in the season, the following experiment was carried out.

MATERIALS AND METHODS

Calves.—Ten Ayrshire calves, about 8 weeks old, were obtained from commercial herds. Careful fecal examination, using the Baermann technique, was made for each calf on 3 occasions prior to the commencement of the experiment. No lungworm larvae were found.

Pasture.—A 4-acre permanent pasture was used. Much of the grass had already been eaten, so concentrate and hay had to be supplied. Each calf was given 2 lb. of rearing concentrate each morning and 2 lb. of hay each evening.

Vaccine.—Five of the calves were given 2 doses of vaccine at a 30-day interval. Each dose consisted of 1,000 3rd-stage larvae which had been subjected to 40,000 r of irradiation by the method described previously.[7] Five calves served as controls.

Parasitology.—Standard McMaster, zinc flotation, Baermann, and worm counting methods were used.

RESULTS

The 10 calves were turned out to graze on the contaminated pasture 20 days after the principals were given their 2nd dose. Clinical signs were noticed 15 days later, and 1 control calf died from severe prepatent parasitic bronchitis on the 18th day. From a portion of 1 lung, 265 immature lungworms were recovered by the Baermann technique. Many more were seen upon histologic examination which also revealed severe pulmonary edema and hyaline membrane formation.

Between the 18th and 20th days, the mean respiratory rates of calves in both groups had risen to almost 70 per minute. That of the vaccinated calves then began to fall and, by day 48, the mean rate for principals was 45, while that of the controls reached a peak of 83 per minute.

The principals were not observed to excrete larvae in their feces at any time during the experiment. On the 30th day, 2 of the 4 surviving controls had counts of 200 and 400 larvae per gram of feces, respectively, and the group mean rose to 500/Gm. on the 44th day (Table 1). One control remained uninfected.

TABLE 1—Group Mean Fecal Counts (Larvae/Gm.) in Calves Vaccinated Twice with Irradiated Lungworm Larvae and Grazed on Contaminated Pasture

	No. of days on pasture					
Group	36	35	37	40	44	49
Controls	162	275	188	287	500	412
Principals	0	0	0	0	0	0

The calves were killed 70 days after being put on pasture, and worm counts on their lungs were carried out. The results are shown (Table 2). Mature worms were not found in the principals; whereas, the controls had a mean burden of 442. Immature worms were found in 2 of the principals.

TABLE 2—Worm Counts at Necropsy of Calves Vaccinated Twice with Irradiated Lungworm Larvae and Grazed on Contaminated Pasture

	No. of worms	
Calf No.	Mature	Immature
CONTROLS		
1	1,250	0
2	0	0
3	367	0
4	150	0
5	.....*	>265
PRINCIPALS		
1	0	0
2	0	70
3	0	0
4	0	0
5	0	55

* Died after 18th day on pasture.

During the whole trial, there was a mean weight gain of 58% in the principals as against 38% in the controls; in the last 4 weeks, the former gained 9% and the latter only 0.7%.

DISCUSSION

One difference between the present double-vaccination experiment and the previous one [3] was that the challenge infection was natural; i.e., it was established by the intake of larvae from grass. This challenge, however, must have come soon after the calves were turned out, as 1 died in the prepatent phase on the 18th day; this is compatible with a massive infection in the first few days. The majority of the calves reacted clinically in the 3rd week, and this, too, points to early ingestion of infective larvae. Four acres is a fairly large field for 10 calves, and it is possible that there was a considerable variation in the numbers of larvae eaten; nevertheless, it does seem unlikely that calf 2, which had neither larvae in its feces nor adult worms in its lungs, did not ingest infective larvae. It is possible that it had some immunity, since detailed history for each animal was not available. The immature worms found in 2 of the principals (7 and 10) may have resulted from ingestion late in the experiment, but it is more likely that they were ingested soon after the calves were placed on pasture and that inhibition of development occurred.[9] This is the product of a balance between degree of immunity possessed by the individual and size of the infecting dose; it has been well documented experimentally in *Nippostrongylus muris* infections.[8] We have noticed it after challenging the immunity of animals vaccinated by a single dose of irradiated larvae but not when double vaccination has been followed by a high (10,000 larvae) challenge. It is therefore likely that these calves (7 and 10) ingested a large number of infective larvae. This is borne out by the clinical findings.

We have noticed regularly[3,7] in experiments and in the field that when vaccinated animals are exposed to heavy infections there is a short sharp rise in respiratory rate. This is due to some larvae reaching the lungs, where the majority die. The degenerating worms induce several lesions, the most important of which in this context is a marked eosinophil-leukocyte reaction which leads to some bronchial plugging and alveolar collapse. This usually passes quickly, and the calves return to normal. Several farmers involved in the field trials thought that parasitic bron-

chitis was incipient in vaccinated calves, but their fears were allayed within a week.

Another factor of possible importance is the interval between vaccine doses. Serologic studies [4,6] of complement-fixing antibody (these do not necessarily correspond with the absolute level of immunity possessed by an animal) have indicated that the highest titer is reached 100 days after primary infection of the susceptible calf. Subsequent reinfections are associated with a secondary response which reaches a peak after about 14 days. Two doses of irradiated larvae with a 6-week interval between do not give rise to a high level of complement-fixation antibody, but the pattern after challenge is that of a full secondary response. In the controlled-dosage challenge experiment with double vaccination,[3] an interval of 42 days was decided upon; whereas, in the present experiment, the time factor necessitated the 2nd dose of vaccine being given 30 days after the first. It is impossible to draw a final conclusion as to the optimal time interval between vaccine doses, as this can be done only when a uniform challenge dose is employed. It may, however, be necessary on individual farms to use the shorter time interval of the present experiment, and it is encouraging that it appears to be associated with considerable protection.

In our earlier report on a trial of this nature,[2] using single vaccination, one significant difference between principals and controls was their fecal larval outputs. This difference was even more marked in the present experiment, since the principals did not have any larvae in their feces on any of the 6 days on which examinations were made. In the field, this must have 2 main consequences: first, the over-all pasture level of infective larvae will be reduced and, in areas where winter survival of larvae on the pasture occurs, it is possible that one of the main sources of new epizootics will be reduced; secondly, the build-up of contamination by autumn-born calves in the following spring will be markedly lowered and the highly susceptible spring-born calves will not be exposed to the high levels of contamination which often cause severe epizootics when mixed grazing is practised.

The total cost of parasitic bronchitis to the farming industry is made up of a number of components; *viz.*, mortality, extra feeding as prophylaxis and treatment; and the lowered growth rate of many affected animals, the latter often being obvious for many months after the acute stage of the disease has passed. The benefit of vaccination in the last respect is underlined in this experiment by the fact that in the last 4 weeks the principals gained a mean body weight of 9%, while the surviving controls gained only 0.7%. In the large field trial of 1958, this factor was evident on farms where stock bulls were being reared for autumn sales; the "normal" check in growth did not take place, and the animals remained on grass when normally they had to be housed.

SUMMARY

Two groups of calves, one of which had been vaccinated twice with irradiated lungworm larvae, were grazed on a pasture on which a severe epizootic of parasitic bronchitis had occurred. There was a marked difference in clinical signs, fecal larval outputs, worm burdens, and growth rates of vaccinated and control calves, in favor of the principals.

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SUMMARIO IN INTERLINGUA

Proba in Pastura, con le Uso de Duo Immunisante Doses de un Vaccino de Bronchitis Parasitic

Duo gruppos de vitellos—le un vaccinate duo vices con irradiate larvas de verme pulmonar, le altere serviente de controlo—esseva ponite in pastura in un campo ubi un sever eruption epizootic de bronchitis parasitic habeva occurrite. Esseva constatate marcate differentias inter le duo gruppos con respecto al signos clinic, al excretion fecal de larvas, al carga de vermes, e al progresso crescential. Omne iste differentias esseva in favor del gruppo vaccinate.

Metabolic Aspects of Ruminant Ketosis

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KETOSIS is a metabolic disturbance which ensues when ketogenesis exceeds ketolysis, so that the concentration of ketone bodies in the blood and urine becomes higher than normal. Ruminant animals have a physiologic ketonemia consisting of β-OH-butyrate [27] which is much higher than that of nonruminant animals in good health; it may not be appropriate to designate this ketosis. Frank ketosis (characterized by readily detectable acetone and acetoacetate, as well as β-OH-butyrate) [27] does not appear during bovine ketosis, ovine pregnancy toxemia, and in fasted pregnant ewes. Ruminant species and their metabolic diseases thus provide an interesting opportunity for the comparative study of ketosis.

The possibility of a carbohydrate shortage being the cause of ruminant ketosis was suggested about 1930 when hypoglycemia was found in ketotic cows and sheep.[6, 22] The clinical belief soon arose that glucose therapy relieved the ketosis and cured the disease in cows but not in sheep. Knowledge gained during 1940–1950 about ruminal [1] fermentation and the intermediary metabolism of volatile fatty acids in normal animals emphasized the rigorous carbohydrate economy of cows and sheep, and strengthened theories of ruminant ketosis based on carbohydrate shortages.[7, 16] Experiments designed to test these theories have been reported during the last 2 or 3 years, and this review is an attempt to orientate these in relation to intermediary metabolism.

A convenient starting point for the consideration of ketosis is the crucial position which acetyl-CoA occupies in metabolism (Fig. 1). Converging upon it are the catabolic pathways of many substances; for example, ruminally produced acetate, butyrate and lactate, and endogenous glucose, fat, and ketone bodies. Leading from it are a limited number of pathways of which the quantitatively important ones are influx into the citric acid cycle, entry into the fatty acid helix, or diversion to ketone bodies. Thus, both anabolism and catabolism of ketone bodies proceed predominantly via acetyl-CoA. The ketone bodies may be regarded as intermediaries intimate with acetyl-CoA, and liable to accumulate when the formation of acetyl-CoA is exceeding its utilization.

To recapitulate, ketosis may be produced when ketogenesis is enhanced, and perhaps, ketolysis is impaired. Both of these events, the 1st in some tissues, the 2nd in others, would be favored by an inadequacy of acetyl-CoA metabolism via the citric acid cycle or the fatty acid helix, or both. Further consideration here will be restricted to these 2 principal pathways of acetyl-CoA metabolism.

THE CITRIC ACID CYCLE

I. The possibility of impaired acetyl-CoA metabolism via the citric acid cycle has been favored by the following observations and associated concepts:

1) Decreased concentrations of blood glucose and liver glycogen are found in ketotic cows and sheep.[6, 13, 22] This may indicate a shortage of carbohydrate in general and oxalacetate in particular.[7, 16, 20, 21] Oxalacetate is required for condensation with acetyl-CoA to form citrate, that is for acetyl-CoA influx into the citric acid cycle (Fig. 2). If it is in short supply, acetyl-CoA could be diverted to form ketone bodies.

Decreased citric acid cycle activity could be associated with low glucose and glycogen concentrations in at least 2 ways: (*a*) Much of the blood glucose and liver glycogen is formed endogenously from propionate and amino acids via nonacetyl-CoA influx into the citric acid cycle. A shortage of such precursors flowing into the citric acid cycle could lead to a shortage of citric acid cycle intermediaries, including oxalacetate, and of glucose and glycogen. (*b*) If for any reason (perhaps a shortage of coenzymes)

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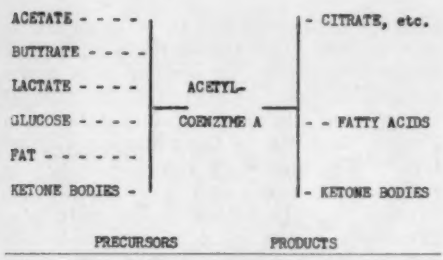


Fig. 1—Examples of precursors and products illustrating the crucial position of acetyl-CoA in ruminant metabolism.

hepatic citric acid cycle activity were to be impaired, it would tend to decrease synthesis of glucose and glycogen despite sufficient precursors.

2) Estimation with labeled glucose of the size of the glucose pool and the over-all rate of glucose metabolism have suggested that these are much less than normal in sheep suffering from pregnancy toxemia.[12] This supports the concept of impaired citric acid cycle activity due to a shortage of cyclic intermediaries more strongly than does unqualified hypoglycemia. These results contrast with those found in ketotic cows (*see* below, II, 2).

3) Glucose, glycerol, propylene glycol, propionate, and adrenocorticoid preparations are used for therapy in bovine ketosis. A common effect would be to prime the citric acid cycle, thus enhancing its activity.

4) Oxalacetic acid administration to fasted pregnant sheep raised the blood glucose concentration and lowered the ketone body concentration.[5] These effects may have been due to oxalacetate entering the citric acid cycle inside cells and increasing its ability to metabolize acetyl-CoA.

5) Concentrations of pyruvate and *alpha*-ketoglutarate were increased while those of citrate and succinate were decreased in the serum of ketotic cows.[1] This suggests that interference with the citric acid cycle is not due to a shortage of oxalacetate but rather to a deficiency in cofactors common to the reactions in which pyruvate and *alpha*-ketoglutarate are converted to citrate and succinate, respectively. The suspected cofactors include coenzyme-A, adenosine triphosphate,[1] and the oxidized form of diphosphopyridine nucleotide.[10]

II. Some experiments which could have substantiated impaired citric acid cycle activity have failed to do so.

1) Oxidation of citric acid cycle intermediaries by liver and brain mitochondria prepared from animals with pregnancy toxemia was normal.[4] These observations render unlikely the possibility that impaired citric acid cycle activity is due to enzyme or cofactor deficiency in ketotic sheep. They in no way preclude, however, impairment due to a shortage of cyclic intermediaries, such as oxalacetate.

2) Estimation with labeled glucose of the size of the glucose pool and the over-all rate of glucose metabolism have suggested that these are not less in ketotic than in normal cows.[14] The low concentration appears to be due to an increase in the volume through which glucose is distributed rather than to a decrease in its amount. Generalization of a carbohydrate shortage, including citric acid cycle intermediaries, during bovine ketosis, is now open to question.

3) Acetate oxidation, estimated by the administration of labeled acetate and the collection of expired $C^{14}O_2$, was not affected when ketosis was induced in sheep.[15]

4) The ratio of expired $C^{14}O_2$ after 1-labeled acetate was administered, to that after 2-labeled acetate, was also not affected by fasting ketosis in sheep.[15] This ratio may be regarded as an indirect index of nonacetyl-CoA influx into the citric acid cycle and, if unchanged, it suggests that

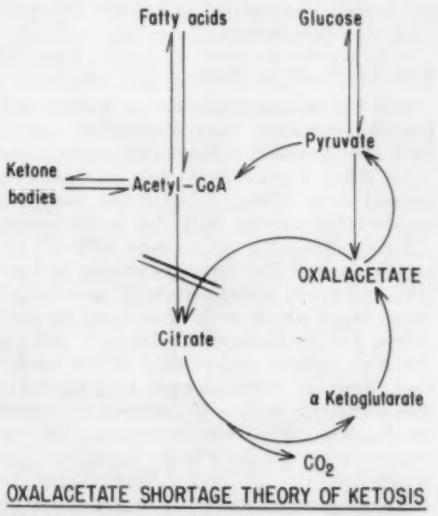
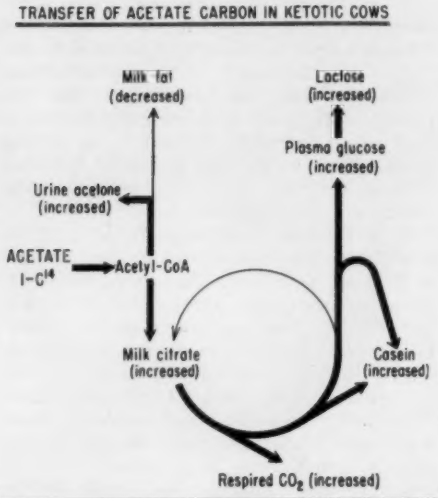


Fig. 2—A scheme showing how a shortage of oxalacetate interfering with entry of acetyl-CoA into the citric acid cycle could lead to ketosis.



—Courtesy American Physiological Society

Fig. 3—A diagram which compares the transfer of C^{14} from acetate to various substances in ketotic cows, to the transfer in normal cows.

the over-all priming of the cycle during ketosis was not less than normal.

5) After acetate-1-C^{14} was injected intravenously, the C^{14} activity of substances derived via the citric acid cycle was greater in ketotic than in normal cows (Fig. 3).[11] This suggests that a greater proportion of the acetate metabolized in ketotic cows proceeds via the citric acid cycle.

THE FATTY ACID HELIX

III. The last-mentioned experiments suggest that impaired lipogenesis from acetyl-CoA may be contributing to ketosis in cows.

1) When acetate-1-C^{14} was injected into normal cows, about 15% of the dose was recovered from the milk fat in 48 hours; the corresponding figure was only 3% in ketotic cows.[11] The data are similar to some obtained from alloxan-diabetic and fasted ketotic rats which were considered to indicate a lipogenic block or lesion.[2] Such a lesion in ketotic cows would divert acetyl-CoA derived from acetate toward entry into the citric acid cycle as well as toward ketogenesis (Fig. 3), integrating an explanation for all the results found in these experiments.

2) Since milk fat synthesis from acetate occurs mainly in the mammary gland, interference with this should result in mammary ketogenesis from acetate. As a test, labeled acetate was injected into the milk cistern of one quarter. The radioactivity of ketone bodies (especially the acetone plus acetoacetate fraction) isolated from the milk of the injected quarter was found to be much greater than that of the noninjected quarters.[10] The mammary transfer of C^{14} to milk ketone bodies and the total C^{14} recovered in milk ketone bodies were progressively higher in a normal, a mildly ketotic, and a severely ketotic cow, while the corresponding parameters for milk fat were progressively lower.[9,10]

3) Preliminary results of the breakdown of milk fat from the above experiments have shown that radioactivity in the water-insoluble, steam-volatile fraction of fatty acids was normally about double that of the shorter chain, water-soluble, steam-volatile, fraction.[26] During ketosis, the radioactivity of the water-insoluble fraction was diminished to about one-fourth its normal value, while that in the water-soluble fraction remained unchanged.[26] These observations are consistent with interference in the fatty acid helix just above B–OH-butyryl-CoA and acetoacetyl-CoA.

4) Although the over-all rate of glucose utilization is unchanged in ketotic cows (*see* II, 2, above), its uptake by different tissues and its flow through different pathways may be altered. For example, there appears to be a decrease in the proportion of glucose traversing the hexose monophosphate (HMP) shunt of ketotic cows.[28] This might well be associated with the lipogenic lesion, since the shunt reduces oxidized triphosphopyridine nucleotide and lipogenesis oxidizes the reduced form (Fig. 4). When a similar association became evident in alloxan-diabetic rats, it was first assumed that the defect in glucose utilization was primary, leading to the lipogenic lesion; at the present time the direction of this cause-effect relationship has become controversial. A decrease of glucose oxidation in the shunt of ketotic cows fits in with concurrent impairment of lipogenesis from acetyl-CoA.

5) When labeled glucose was injected intravenously into ketotic cows, the specific activity of many substances was lower than normal.[20] The specific activity of citrate was less than that of glycerol, possibly due to interference in some reaction between glycerol and citrate, a concept reminiscent of that derived from the high pyruvic concentration and low citric concentration in

the serum of ketotic cows.[1] A vulnerable step may be in the oxidative decarboxylation of pyruvate to form acetyl-CoA, a reaction requiring oxidized diphosphopyridine nucleotide which may come into short supply when there is a lipogenic lesion. This association is similar to that proposed between lipogenesis and the HMP shunt, except that diphosphopyridine nucleotide is the coenzyme involved, instead of triphosphopyridine nucleotide (Fig. 4).

Lower specific activities after the administration of labeled glucose, might also be due to the entry of greater amounts of carbon into the sampled substances from precursors other than glucose. If more acetyl-CoA derived from mobilized body fat or diverted acetate was entering the citric acid cycle, it would tend to lower the specific activity of citrate more than that of such substances as protein, glycerol or lactose. This is precisely what was observed.[28]

The results obtained with labeled glucose and with labeled acetate complement one another, and from them we can draw 2 conclusions: (*a*) mammary lipogenesis from acetyl-CoA is impaired during bovine ketosis; (*b*) a higher proportion of the acetate metabolized and, perhaps, a lower proportion of the glucose metabolized proceed via acetyl-CoA into the citric acid cycle in ketotic cows.

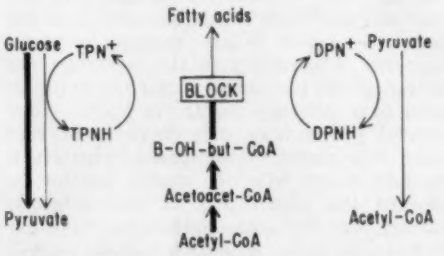
IV. A few observations which have been interpreted in the past in other ways may seem to require resolution with a lipogenic lesion.

1) Fatty livers found in ketotic cows and sheep have suggested that body fat is mobilized and that hepatic ketogenesis is enhanced. This adaptation has been traditionally regarded as providing alternative substrates for oxidation when that of carbohydrate is diminished.

Many aspects of this classical concept are currently in need of revision. First, the liver may not be the dominant, let alone the sole, site of ketogenesis in cows and sheep. The rumen epithelium can convert butyrate to ketone bodies,[18] the udder can incorporate acetate-C^{14} into acetone and acetoacetate,[10] and even the tissues of the head appear to contribute ketone bodies to the blood after butyrate administration.[8] The relative importance of hepatic and extrahepatic ketogenesis in health and disease is not yet decided. However, the recent demonstration that perfused livers taken from ketotic cows produce negligible amounts of ketone bodies shifts emphasis to extrahepatic tissues.[17] Second, oxidation may not be the dominant or only fate of fat mobilized during disease. Thus, if a disease involves decreased oxidation of glucose, oxidation of mobilized fat may increase, in accord with the classical competition theory. Similarly, in a disease which involves impaired lipogenesis from acetate, lipogenesis from alternative substrates arising from mobilized fat may also increase.[11]

2) Acetylation of sulfonamides was reported to be less in ketotic than in normal cows.[19] Diminished acetylation was attributed to a shortage of acetyl-CoA, due to inadequate activation of acetate, due to a shortage of adenosine triphosphate, due to diminished citric acid cycle activity, due to a shortage of oxalacetate.[19] A possible flaw in this logical sequence is the assumption that the acetyl-CoA involved is derived from acetate. Indeed, the acetylation of sulfonamides is thought to occur in the liver, an organ which appears to take up little if any acetate.[25] The acetyl-CoA involved may alternately be coming from pyruvate and be in short supply if there is interference in the oxidative decarboxylation of pyruvate. This is consistent with the accumulation of serum pyruvate and the glucose-C^{14} experiments in ketotic cows. Oxidative decarboxylation could be linked with lipogenesis through diphosphopyridine nucleotide.

3) The effectiveness of a given therapy has been thought to substantiate its rationale, although different authors have thus justified different rationales. For example, adrenocorticoid therapy has been argued to alleviate adrenal insufficiency,[24] restore



LIPOGENIC BLOCK THEORY OF BOVINE KETOSIS

Fig. 4—A scheme depicting possible interrelationships between fat and carbohydrate metabolism by way of coenzymes, and how a lipogenic lesion could lead to ketosis.

liver glycogen,[23] or prime the citric acid cycle.[34] We should also consider other possible beneficial effects, insulin antagonism, and reduction of oxidized pyridine nucleotides. This example suggests that clinical effectiveness of a given therapy neither shows that a single effect is the only beneficial one, nor precludes the admission of more than one rationale.

CONCLUSIONS

The simple concept of a carbohydrate shortage as the cause of ruminant ketosis appears to be standing experimental tests better in ovine pregnancy toxemia than in bovine ketosis. Nearly all observations on ketotic sheep remain consistent with impairment of citric acid cycle activity due to a shortage of oxalacetate, an exception being the preliminary report of experiments with labeled acetate.[15] Any impairment of citric acid cycle activity in ketotic cows, on the other hand, appears more likely to be due to a shortage of enzymatic cofactors,[1, 10] and to be less important, at least in acetate metabolism, than impaired lipogenesis.[11]

Finally, let us briefly consider the significance in veterinary medicine of research on metabolic aspects of ruminant ketosis. A working plan for the pathogenesis of diseases such as ovine pregnancy toxemia and bovine ketosis is this: Any one of a number of factors which in health are in harmony may change so as to initiate a common, sequential pattern of endocrine and metabolic derangements, some of which become clinically manifest. Ketosis serves as a portal of entry for investigation into the hidden pattern of abnormal metabolism. In the opinion of the author, the most successful therapy will eventually be designed to correct the multitude of derangements which have developed before therapy is commenced. A corollary to the working plan is that at the beginning of the common pattern is a primary metabolic lesion. Successful prophylaxis may have to prevent only this single derangement, when it is found! Meanwhile, the search has for its reward the elaboration of knowledge of metabolism, for some pathways which are difficult to detect in health become quantitatively obvious during disease.

SUMMARY

Recent observations on ketotic cows and sheep by different groups of investigators using liver biopsy, estimation of serum intermediaries, mitochondrial oxidation and labeled acetate and glucose may be interpreted in relationship to the 3 principal pathways open to acetyl-CoA: influx into the citric acid cycle, entry into the fatty acid helix, or diversion to the ketone bodies. Most observations on ketotic sheep are consistent with impaired citric acid cycle activity being due to a shortage of cyclic intermediaries, particularly oxalacetate. In ketotic cows, however, any impairment of citric acid cycle activity appears more likely to be due to a shortage of enzymatic cofactors, and to be more remote than impaired lipogenesis from the elusive primary metabolic lesion.

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SUMMARIO IN INTERLINGUA

Aspectos Metabolic de Cetosis in Ruminantes

Recente observationes in vaccas e oves cetotic per differente gruppos de investigatores, usante biopsia hepatic, estimation de intermediarios seral, oxydation mitochondrial, e marcation de acetato e glucosa, pote esser interpretate in relation al tres principal circuitos disponibile a acetyl-coenzyma A: Influxo ad in le cyclo de acido citric, entrata ad in le helice de acido grasse, o diversion verso le corpores de cetona. Le majoritate del observationes in oves cetotic es compatibile con le these que un defecto in le activitate del cyclo de acido citric es le resultato de un deficientia de intermediarios cyclic, particularmente oxaloacetato. In vaccas cetotic, del altere latere, il pare plus probabile que defectivitate in le activitate del cyclo de acido citric es le effecto de un deficientia de cofactores enzymatic e que illo es plus remote que defectos de lipogenese ab le elusive lesion metabolic primari.

Nocardia asteroides as a Mammary Pathogen of Cattle. I. The Disease in Cattle and the Comparative Virulence of 5 Isolates

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Davis, California, and Honolulu, Hawaii

EARLY IN 1957, an unusually tenacious form of mastitis appeared in a dairy herd located in the southern coastal area of California. The disease was marked by high elevations in the body temperature and extensive fibrosis of the infected glands. The use of antibiotics and chemotherapy had failed to alleviate the condition. *Nocardia asteroides* was isolated from the milk of 28 of 157 animals. The organism grew in pure culture from 19 of 40 culturally positive milk samples obtained from clinically diseased glands. These findings were the subject of earlier reports.[21, 22]

A review of the literature disclosed that while *N. asteroides* has been reported from infections of both man and animals, these infections are not common. The organism had been isolated from 2 cattle with mastitis, but neither of these isolations was made from cattle in the United States nor was more than a single animal involved in either instance. Reports of the organism's thermal resistance and its infectivity for man suggested that public health implications might arise from this disease in cattle.

Since our first recognition of the infection, we have diagnosed this disease in 9 additional dairy herds. It is the purpose of this report to describe the infectious agents isolated from 7 infected herds and the disease they caused in naturally and experimentally infected cattle.

The recent literature contains a series of articles of veterinary interest concerning canine,[3, 4, 6, 16, 18, 26, 27] feline,[16, 26] and equine [1] nocardial infections. A review of veterinary literature revealed 2 cases of bovine mastitis caused by *N. asteroides* that were reported prior to 1957; 1 was from Australia,[19] the other from Canada,[2] and both were single-quarter infections. The reports of the infection in California marked recognition of the infection in epizootic proportions and stimulated speculation as to the significance of this disease. Subsequent to this diagnosis, we studied the infection in 6 dairy herds in California and 3 in Hawaii.* The disease has also been recognized in Texas,[15] Alabama,** and Massachusetts [14]; reports of nocardial mastitis of the goat in Africa [8] and of the cow in Canada [9] have recently been published, giving the problem even greater geographic and statistical dimensions.

MATERIALS AND METHODS

Isolation and Identification of Nocardia asteroides.—In the taxonomy of the genera Mycobacterium, Nocardia, and Streptomyces, it is often difficult to locate reliable criteria of differentiation and impossible to locate unanimity of nomenclature.[11, 24] Perhaps this is best explained by the fact that the nocardias are transitional forms between the non-acid-fast, filamentous Streptomycetes and the acid-fast mycobacteria. Gordon and Mihm [10] described sporulation of *N. asteroides* indistinguishable from that of Streptomyces, while Cutino and McCabe [7] have described a species of Nocardia which produces essentially glabrous, nonfilamentous colonies typical of the mycobacteria. An extensive study of carbohydrate utilization by species of Nocardia did not establish reliable criteria for the separation of these species.[17] The scheme of biochemical and morphologic reactions proposed by Gordon and Smith [12] did not completely separate Streptomyces from Nocardia when additional strains were subsequently tested.

Identification procedures utilized in this study were taken in part from the work of Gordon *et al.*,[11] of Schneidau and Shaffer,[24] and those used at the mycology section of the Communicable Disease Center, U.S. Public Health Service. The identity of strains isolated from each of the 7 herds included in this study were confirmed at the Mycology Laboratory, Communicable Disease Center, USPHS, Chamblee, Georgia.

Isolations of *Nocardia asteroides* were made by streaking aseptically collected milk samples on tryptose agar plates containing 5% washed bovine

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* Two of the epizootics in Hawaii were recognized early in 1957.

** Dr. C. S. Roberts, State Veterinary Diagnostic Laboratory, Auburn, Ala., 1958.

erythrocytes. The use of fresh milk was preferred to incubated milk, as the overgrowth of bacterial residents in incubated samples often masked the nocardial growth. These plates were incubated aerobically at 37 C. for 72 to 96 hours. Observations for colony color and the presence of aerial myceliums were made from 14- to 28-day-old cultures on Sabouraud's dextrose agar slants. Mycelium formation, branching, and fragmentation were observed on 14- to 20-day-old slide culture preparations, using blocks of potato dextrose agar as the nutrient medium.

Acid-fast staining determinations were made on exudates from experimentally infected guinea pigs. Kinyoun's acid-fast technique with 2% sulfuric acid (alcohol-free) as the destaining reagent was used.

The ability of the isolate to utilize paraffin as a carbon source was tested after the method of Frey and Hagan[10] by emulsifying colony isolates from agar slants in Sohngen's liquid medium, adding paraffin-coated glass rods, and incubating at 37 C. for 28 days. The ability of the organism to grow at 46 C. was determined on nutrient gelatin plates incubated at 25 C. for 14 days. The ability to hydrolyze casein was tested on 5% skim milk agar incubated at 25 C. for 14 days; casein hydrolysis appeared as a clearing of the medium about the colonies. The hemolytic activity of each isolate was determined on tryptose agar containing 5% washed bovine erythrocytes. Two methods were utilized to determine the reduction of nitrates to nitrites; both nitrate broth (Difco) cultures, incubated for 48 hours at 37 C., and nitrate agar (Difco) slants, incubated for 10 days at 37 C., were tested for nitrate reduction by the addition of sulfanilic acid and α-naphthyl amine. Uninoculated medium controls were also incubated and tested.

Pathogenicity for guinea pigs was determined by 2 methods. A single colony, picked from a plate 48 hours old, was emulsified in 2.0 ml. of 1% serum broth and incubated for 48 hours at 37 C. Plate counts revealed that such broth cultures contained approximately one billion organisms per milliliter. One milliliter of these broth cultures was inoculated intraperitoneally into guinea pigs weighing approximately 400 Gm. Additional studies of pathogenicity for guinea pigs were made by preparing inoculums from saline dilutions of serum broth cultures. When it was found that clumping of the organisms interfered with accurate dilutions, the culture was agitated for 15 minutes in a tissue disintegrator cup* containing sterile glass beads. Inoculation dilutions and plate counts were then made from the resulting culture suspension. The inoculated animals were observed daily for 14 days. Animals dying during that period were examined and representative lesions were cultured. Animals surviving for 2 weeks were subjected to euthanasia and similarly examined. Strains of Nocardia not producing death or satisfactory lesions in guinea pigs were inoculated into additional animals, using 1.0 ml. of a 48-hour serum broth culture and 1.0 ml. of 5% gastric mucin as the intraperitoneal inoculum.

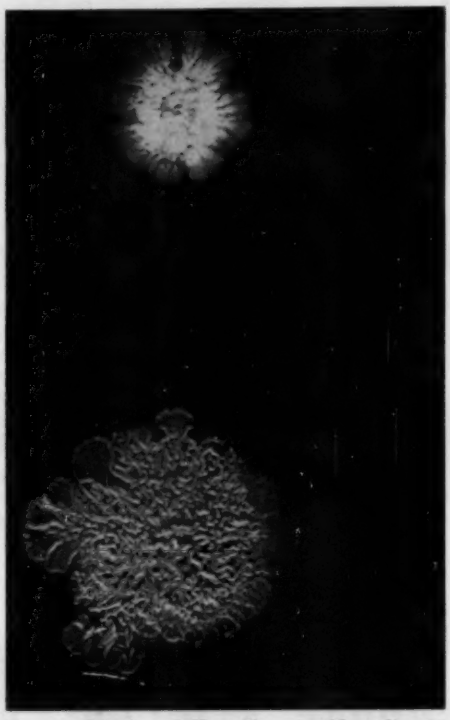

Fig. 1—Colonies of Nocardia asteroides after 28 days of incubation. The uppermost colony produced aerial myceliums; the colony below was entirely glabrous.

Studies of Naturally and Experimentally Infected Cattle.—The studies on naturally infected cattle and isolates from these animals were conducted in the following 7 commercial dairy herds:

Herd A.—This herd, located in the south coastal area of California in San Luis Obispo County, contained 157 cows of the Guernsey and Holstein-Friesian breeds. The dairy produced market milk, and the cows were milked by machine. The cattle were maintained on pasture throughout most of the year, although feeding corrals were used during the winter months. A group of cows, designated as the hospital string, was maintained separately. This group was comprised of cows to be treated during the dry or nonlactating period, those with active cases of mastitis, those sick with other diseases, and cows still in the stage of colostrum secretion following parturition. The herdsman treated all cows by intramammary infusion during the dry period.

Herd B.—This herd, located in the central valley

* Mickle Tissue Disintegrator, produced by H. Mickle Co., Gomshall, Surrey, England.

region of California in Kings County, was comprised of 48 Holstein-Friesian cows. The dairy produced market milk. The cows were milked by machine and were maintained on pasture. A program of treating mastitis by intramammary infusion during the dry period was practiced.

Herd C.—This herd, located in the northern valley region of California in Glenn County, was comprised of 40 Holstein-Friesian cows. The dairy was maintained and produced milk for a monastery. The cows were milked by machine and were maintained on pasture. Treatment for mastitis was made regularly during the dry period. Treatments were administered by the herdsman.

Herd D.—This herd, located in the northern Sierra foothill region of California in Placer County, was comprised of 46 registered Guernsey cows. The dairy produced market milk. The cows were milked by machine and were maintained on pasture. Intramammary infusions for mastitis were not on a programmed basis and were performed by a hired milker.

Herd E.—This herd, located in the northern Sierra foothill region of California in Placer County, was comprised of 70 cows of Guernsey and Brown Swiss breeding. The dairy produced market milk. The cows were milked by machine and were maintained on pasture. Intramammary infusion treatments for mastitis were not conducted on a programmed basis and were performed by the owners.

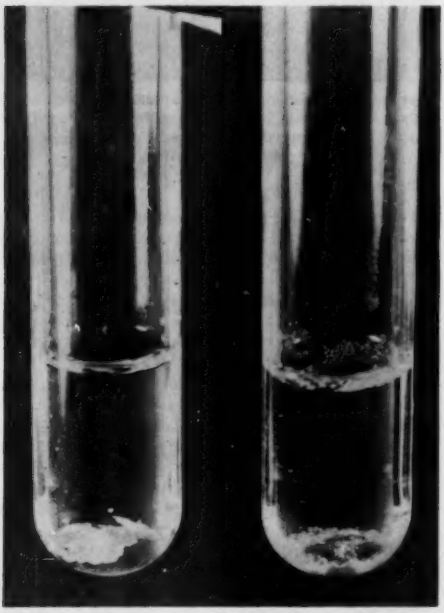

Fig. 2—Serum broth cultures of Nocardia asteroides following 48 hours of incubation. The tube at the left contains fluffy mycelian masses and flakes of the pelicle which have settled to the bottom. The tube at the right has mycelian plaques at the surface.

Herd F.—This herd, located in Hawaii on the island of Oahu, was comprised of approximately 700 cows of Holstein-Friesian and Guernsey breeding. The dairy produced market milk and the cows, milked by machine, were maintained in corrals where they were fed concentrates and chopped fodder. The cattle had been moved to newly prepared, modern premises 8 months prior to our investigation. A separate hospital barn was maintained for cows being treated for mastitis in the dry period, those with clinical mastitis and other diseases, and those in the stage of colostrum secretion following parturition. Intramammary infusion treatments for mastitis were used on a regular basis during the dry period. All treatments were performed by hired milkers at the direction of the herdsman.

Herd G.—This herd, located in Hawaii on the island of Oahu, was comprised of approximately 500 cows of the Guernsey and Holstein-Friesian breeds. The dairy produced market milk and the cows, milked by machine, were maintained in corrals where they were fed concentrates and chopped fodder. A separate hospital barn was maintained as described for herds A and F. Intramammary infusion treatments for mastitis were used on a regular basis during the dry period and for clinical cases during lactation. Treatments were performed by hired milkers at the direction of the herdsman.

The presence of *N. asteroides* as a true invader of the mammary glands was verified by repeated cultural isolations. Demonstration of the organism in histologic sections and by cultural isolation was made from tissues removed at necropsy of cows from herds A, E, F, and G.

With the exception of herds F and G, which were studied in Hawaii, milk from each lactating cow in each herd was cultured to determine the incidence of infection. When nocardial isolations were made, another sample was obtained, the isolation was verified, and the infected mammary quarters were identified. In herds F and G, the lactating cows were screened for mammary inflammation by means of the California mastitis test (CMT)[20] or the Whiteside test,[20] and reacting cows were selected for cultural examination. In neither of these herds was the incidence of infection derived from a culture analysis of the entire herd.

Estimated total losses in each herd were based on records kept by each herdsman and should be considered a close estimate of total losses due to nocardial mastitis. The characteristics of infected udders were sufficiently distinct that once the causative agent was known the herdsman rarely missed a culturally positive case of long duration.

The naturally occurring disease was studied in herds A, D, E, and F, in which the age of infected animals, the stage of lactation, the characteristics of clinical disease, the previous mastitis history, and treatment procedures were recorded. Infected

TABLE 1—Cultural Reactions of 7 Herd Isolates of Nocardia asteroides of Bovine Mammary Origin

Herd	Colony color	Aerial mycelium	Mycelium		Acid-fastness	Paraffin utilization	Growth at 46 C.	Gelatin liquefaction	Casein hydrolysis	Hemolysis	Nitrate reduction	Pathogenicity for guinea pig
			Branched	Fragmented								
A	Buff-orange	—	+	+	+	+	+	—	—	—	+	+
B	Buff-orange	—	+	+	+	+	+	—	—	—	+	+
C	Buff-orange	—	+	+	+	+	+	—	—	—	—	+
D	Buff-orange	—	+	+	+	+	+	—	—	—	+	+
E	Buff-orange	—	+	+	+	+	+	—	—	—	+	+
F	Chalky white	+	+	+	+	+	+	—	—	—	+	+
G	Buff-orange	—	+	+	+	+	+	—	—	—	+	+

cows were isolated and observed daily for signs of clinical illness. Animals with repeated culturally positive milk and typical signs of infection were selected for necropsy and cultural examination from herds A and F.

Experimental infections were attempted in lactating dairy cattle shown to be free of the disease by repeated preinoculation cultural examination of their milk. After complete milking of the quarter to be infused, the teat orifice was carefully disinfected and saline suspension of serum broth cultures of known age was infused into the prepared gland. Inoculum preparations and postinoculation milk samples, examined culturally and microscopically, verified the absence of contaminating organism populations. In general, each inoculum contained from 1 to 20 billion viable mycelian fragments. After infusion, the inoculated gland was massaged to distribute the inoculum, and the gland was not milked for 24 hours in order to allow the parasite a better opportunity to establish itself. Daily rectal temperature, CMT reactions, and clinical observations were recorded for each experimentally infected cow. Weekly milk samples from each mammary quarter were examined culturally, and periodic, hematologic, and serologic observations were made.

RESULTS

CULTURAL IDENTIFICATION

Colonies of *N. asteroides* developed slowly on tryptose blood agar and were seldom noticeable prior to 48 to 72 hours of aerobic incubation at 37 C. After 72 hours, the colonies were about 1 mm. in diameter, raised, opaque, whitish, and firmly adherent to the mediums. Microscopic examination of the colony revealed a filamentous structure. Additional incubation produced colonies with slightly granular surfaces and translucent mycelioid edges. Pigmentation of the colonies usually appeared by 72 hours; it varied from initial light buff to bright salmon by the 4th week of incubation. The formation of aerial myceliums was observed in only 1 strain and was always present in the isolates from that herd; the isolates from the remaining herds were entirely glabrous (Fig. 1). The organisms did not hemolyze bovine or equine erythrocytes.

The organisms had fine mycelian elements 1 μ or less in thickness. Branching filaments, as well as shorter rod and coccoid forms, were evident.

In serum broth, a delicate waxy pellicle was formed from discrete mycelian plaques which grew on the surface after 48 hours of incubation. This pellicle was slightly adherent to the sides of the tube and was easily disrupted when shaken. Small, round, fluffy, mycelian masses formed at the bottom of the tube; the intermediate zones were clear (Fig. 2). One strain, that which produced aerial myceliums, peptonized and darkened the broth medium.

The ability to utilize paraffin as a source of carbon was consistent with all strains. The reaction was seen by the growth of small filamentous colonies which adhered to the paraffin-coated rod. This reaction was useful when isolating the organisms from soil and other contaminated specimens, as described by Frey and Hagan.[27]

The cultural reactions important for classification are listed (Table 1) for isolates obtained from 7 infected herds.

GUINEA PIG INOCULATION

In addition to the cultural methods described, the demonstration of animal pathogenicity was considered mandatory for positive identification of *N. asteroides*. Serum broth cultures, containing approximately one billion viable mycelian element per milliliter, were injected intraperitoneally into guinea pigs. Guinea pigs

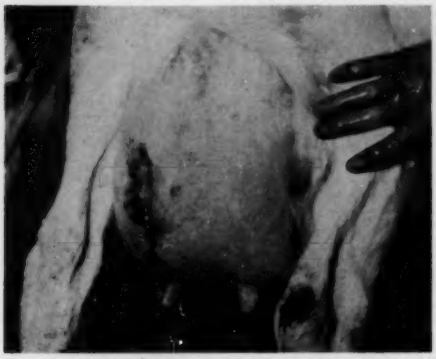

Fig. 3—Draining sinus tract formation following acute nocardial mastitis. Experimental infection; cow 329.

given inoculations of strains A, B, F, and G all died in 3 to 9 days, a shorter period than is usually observed in this infection.[5] The remaining 3 isolates did not regularly cause death of the animals up to 2 weeks following inoculation, but lesions were present from which *N. asteroides* was isolated when the animals were killed and examined at the end of this period. The use of 5% gastric mucin in equal parts with 48-hour broth cultures or suspensions of growth from solid mediums enabled these 3 strains to kill the experimental animals within 5 to 14 days.[25]

Necropsy of infected guinea pigs revealed scattered to abundant raised, white lesions, 1 to 2 mm. in diameter, on the parietal and visceral peritoneum. These foci were hard and caseous. Larger lesions were often encountered at the inoculation site. Acid-fast filaments were demonstrated in these foci.

The virulence in guinea pigs was further tested by varying the numbers of viable mycelian fragments in the inoculum. These results are shown (Table 2).

TABLE 2—Guinea Pig Inoculation—Response to Varied Doses of Nocardia asteroides Injected Intraperitoneally

Nocardia isolate inoculated	No. of organisms inoculated											
	10^6			10^7			10^8			10^9		
	Neg.	Pos.	Died	Neg.	Pos.	Died	Neg.	Pos.	Died	Neg.	Pos.	Died
A	0	2	5	..	..	..	..	..	..	0	0	3
C	3	1	0	2	0	0	..	..	..	0	5	0
D	..	..	..	4	0	0	8	0	0	4	7	3
F	2	2	0	..	..	..	0	2	2	0	0	5

Neg. = Animal lived and had no lesions at necropsy 14 days after inoculation. Pos. = Animal lived, and culturally positive lesions were present at necropsy. Died = Animal died within 14 days with culturally positive lesions at necropsy.

THE DISEASE IN CATTLE

The Clinical Syndrome of Natural Infection.—In all herds, infections of the mammary gland due to such common bacterial pathogens as streptococci, staphylococci, pseudomonads, and coliform bacilli were culturally detectable. The clinical syndrome of the animals shedding *N. asteroides*, however, whether in pure culture or in combination with other organisms, was distinct in herds A, B, F, and G. The syndrome of acute onset and severe clinical course was constant in these 4 herds. In the remaining 3 herds, the response of infected cattle was less pronounced, being essentially a mild chronic involvement of the mammary gland.

Clinical evidence of infection was most frequently encountered 2 to 10 days after parturition, nearly all cases occurring between the last month of gestation and the 2nd month of lactation. The most severe cases observed were those that occurred within 1 or 2 days of parturition.

In cases occurring at parturition, febrile reaction of 104 F. and above was usually observed during the first 48 hours of lactation. The rectal temperature often increased to 107 or 108 F. and was maintained at high levels for a period of several weeks. When the acute clinical stage passed and became chronic, the body temperature often dropped to nearly normal levels. Infected animals were extremely depressed and anorexic; lacrimation and salivation often occurred during the acute clinical period. Loss of body weight and condition was marked.

The involved mammary glands rapidly enlarged and assumed a firmly fibrosed

"woody" texture. Occasionally, discrete nodules 2 to 5 cm. in diameter were palpable from the exterior. Mammary exudate from such glands was typically whitish and viscid, and it often contained discrete blood clots and small white granules 1 mm. in diameter. Microscopically, these granules were composed of masses of myceliums. These glands often formed small, draining, sinus tracts (Fig. 3) which liberated typical exudate and mycetoma granules; on occasion, the gland ruptured (Fig. 4). Following drainage of the gland, the body temperature rapidly fell toward normal and the cows usually resumed eating; in the absence of such relief, they often died in 2 to 4 weeks.

In herds C, D, and E, signs of systemic illness were either absent or confined to a moderate febrile response following parturition. Most frequently, evidence of such infections were restricted to mammary inflammation, the shedding of clots of exudate in the milk, or the production of secretions reacting strongly to the CMT. The development of palpable fibrosis ensued in such glands.

The type of clinical disease was a constant feature within the different herds studied and appeared to be related to the virulence of the nocardial isolate. The clinical type of infection, the known number of infected animals as determined by cultural diagnosis, and the estimated total losses through death or culling of animals presumed to be infected in the 7 herds are presented (Table 3), along with the results of intraperitoneal inoculation of guinea pigs. From these reactions and the responses of guinea pigs to varied doses of each isolate (Table 2), the relationship between virulence of organism and severity of infection in dairy herds was established. This relationship was further explored during studies of experimental infection of cattle.

A severely affected 1st-lactation heifer which had had clinical evidence of infection since her 1st week of lactation was selected from herd A for necropsy on the basis that pathologic changes present in the mammary gland would represent those due to nocardial infection only. *Nocardia asteroides* had been recovered from the udder in pure culture on 3 consecutive occasions; 3 of the mammary quarters were involved. Grossly, the udder contained areas of diffuse fibrosis in the dorsoposterior regions of the right rear quarter. Several palpable, thick-walled

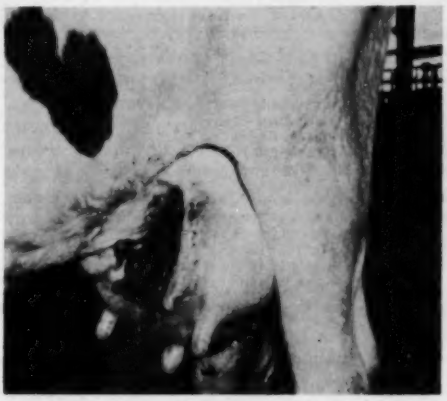

Fig. 4—A ruptured mammary quarter following acute nocardial mastitis.

abscesses, 2 to 5 cm. in diameter, were present in the lower portions of the gland. Lesser granulomatous lesions were scattered throughout the secretory tissue. The lining of the teat sinus, gland cistern, and lower collecting ducts was greatly thickened and rough, presenting a granular appearance. The lining of higher ducts was similarly involved in various areas. The discrete pathologic changes seen in this udder have been previously described.[22]

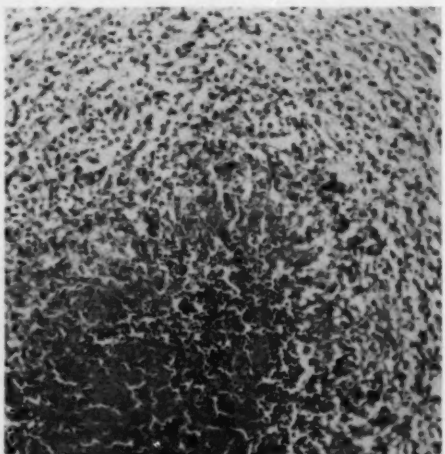

Fig. 5—Photomicrograph of an encapsulated granulamatous lesion in infected mammary tissue of cow 3107, experimentally infected with Nocardia. This section was made 1 month postinoculation. H & E stain; x 55.

TABLE 3—Herd Incidence of Nocardial Mastitis and Clinical Type of Infection

Herd	Location	Total herd	Cultural isolates	Deaths*	Total losses*	Clinical type	Response of guinea pigs to intraperitoneal inoculation of 1 billion organisms
A	South coastal California (San Luis Obispo Co.).	157	28	2	56	Severe.	Died in 4 to 8 days.
B	South central valley, Calif. (Kings Co.).	48	2	1	2	Intermediate to severe.	Died in 4 days.
C	Northern valley, Calif. (Glenn Co.).	40	3	0	..	Chronic.	Required gastric mucin plus culture to kill.
D	Northern Sierra foothills, Calif. (Placer Co.).	46	8	0	12	Intermediate to chronic.	Died in 1 to 2 weeks or required gastric mucin to kill.
E	Northern Sierra foothills, Calif. (Placer Co.).	70	7	0	10	Chronic.	Required gastric mucin plus culture to kill.
F	Hawaii, Island of Oahu.	700	43	19	300	Severe.	Died in 4 days.
G	Hawaii, Island of Oahu.	500	....	80	180	Severe.	Died in 9 days.

* Attributed to *N. asteroides* infection by herd manager.

The distribution of lesions in the glandular tissue and the relative age of 1 pathologic process to another suggested an ascending lobular involvement; 1 lobule was found to have little or no pathologic change, while the tissue of an adjacent lobule was nearly unrecognizable due to the destruction of its secretory elements.

The pathologic changes observed in this cow were typical of those seen in 8 naturally infected cows examined at slaughter. In some udders, the fibrosis was more diffuse and the lesions were generally smaller; in others, the necrotic changes of the parenchyma were so extensive that only a framework of fibrous tissue surrounding large pockets of exudate remained. These latter lesions were found to result from mixed infections with *N. asteroides* and *Pseudomonas aeruginosa* or *Corynebacterium pyogenes*. In 2 cattle with field cases of over 1 month duration, firm, caseous foci measuring 2 to 3 mm. were found in the lungs. These lesions, from which *N. asteroides* was isolated, were uniformly distributed throughout the pulmonary parenchyma rather than following the bronchial tree. This distribution and the uniformity of size of the lesions suggested hematogenous metastasis.

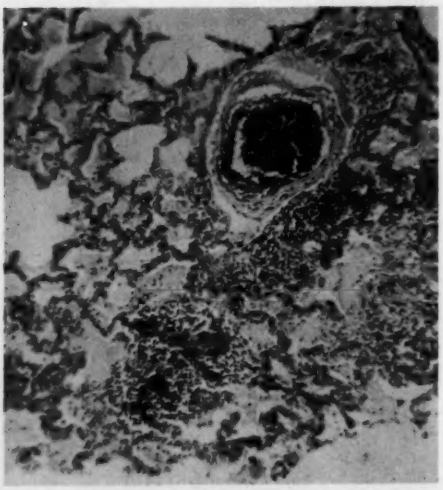

Fig. 6—Photomicrograph of lung lesion of cow 52. Congestion of the capillary bed surrounds a lesion below and to the left of the pulmonary vessel. The gray, vermiform figure in the center of the lesion is an infective embolus which filled the capillary space and is surrounded by leukocytic infiltration. H & E stain; x 55.

Experimental Infection of Dairy Cattle.—In order to study the reproducibility of the infection, to compare the degree of response to infection with different isolates, and to observe the sequence of the clinical signs from the time of infection, 14 mammary quarters of 7 experimental cows were inoculated. Isolates from herds A, D, F, and G were used. Clinical and laboratory observations made during each experimental infection are included in the following discussion.

Cow 3107.—A 1st-lactation animal with no previous history of infectious mastitis was inoculated in 2 mammary quarters with broth cultures of *N. asteroides* isolated from herd A. The right rear quarter was given approximately 10 billion organisms; the left front quarter was given approximately 10 million organisms. Control infusions of sterile broth were made into the remaining quarters.

Twenty-four hours later, the quarters inoculated with nocardial cultures produced visibly altered milk.

By the 2nd day postinoculation, the cow's body temperature was 104.8 F. Milk

from both inoculated quarters contained strands of exudate, while the 2 control quarters gave milk which appeared normal but reacted positively with the CMT. Total milk yield was considerably reduced.

By the 5th day, both inoculated quarters were enlarged and hard; the cow had a body temperature of 106.6 F. and was depressed and inappetent. Fearing a fatal termination, 500 mg. of novobiocin was infused into the left front quarter and 200 ml. of 25% sulfamerazine solution was administered by venoclysis at 24-hour intervals for 3 days. The body temperature fell to 101.8 F. The febrile reaction subsequently returned and remained until the cow was killed 33 days postinoculation. *Nocardia asteroides* was isolated from both inoculated quarters during this period.

Necropsy findings in cow 3107 were similar to those in cattle with field cases. The glands were enlarged and hard; extreme fibrosis with intervening necrotic areas was apparent grossly. Two adjacent foci appeared to have penetrated the intermammary septum and invaded a noninoculated quarter. However, at no time was the organism cultured from the milk of this quarter. The linings of the teat sinuses were thickened and granular. Microscopic examination revealed an epithelial hyperplasia of the sinus and ducts, but the organism was not seen in these tissues. Discrete, encapsulated, granulomatous lesions were microscopically visible, within which Nocardia could be demonstrated (Fig. 5). Isolated lobules of nearly normal glandular tissue were encountered.

TABLE 4—Total and Differential Leukocyte Counts Following Experimentally Reproduced Nocardial Mastitis

	Cow No.				
Cells (%)	3107	328	329	119	52
PREINOCULATION					
WBC/cmm.	....	11,550	5,500	5,000	....
Band	....	0	0	0	....
Neut.	....	34	16	29	....
Lymph.	....	51	79	54	....
Mono.	....	3	2	4	....
Eos.	....	12	3	13	....
POSTINOCULATION					
	5 days	7 days	7 days	7 days	Field infection 2 weeks postpartum
WBC/cmm.	8,250	5,700	9,350	6,200	4,000
Band	2	0	0	22	2
Neut.	40	21	50	56	43
Lymph.	43	68	43	17	39
Mono.	2	6	3	4	10
Eos.	13	5	4	....	6
	7 days	14 days	14 days	14 days	
WBC/cmm.	8,200	8,050	9,550	22,450	....
Meta.	0	0	0	2	....
Band	0	4	2	11	....
Neut.	60	65	40	76	....
Lymph.	35	30	56	9	....
Mono.	4	1	1	2	....
Eos.	1	0	1	0	....
	10 days	21 days	21 days	21 days	Experimental plus field infection 10 days postpartum
WBC/cmm.	8,350	....	....	....	7,050
Meta.	1	0	0	....	0
Band	6	2	0	....	13
Neut.	58	34	23	....	49
Lymph.	30	55	69	....	37
Mono.	5	6	7	....	1
Eos.	....	2	1	....	....
	13 days				
WBC/cmm.	10,250	....	....	....	....
Meta.	0	....	....	....	....
Band	8	....	....	....	....
Neut.	59	....	....	....	....
Lymph.	31	....	....	....	....
Mono.	1	....	....	....	....
Eos.	1	....	....	....	....

Cow 3032.—A 3rd lactation animal, producing 20 lb. of milk per day, was inoculated by intramammary infusion with approximately 100 billion organisms in the right front quarter and in the left rear quarter using strains of *N. asteroides* from herds G and D, respectively. Within 48 hours of inoculation, milk production had decreased to 10 lb. The right front quarter was swollen and produced a thickened, viscid secretion, while the left rear quarter developed only slight firmness. Rectal temperature rose to 105.1 F. by the 4th day postinoculation. The right front quarter became progressively enlarged and hard; the left rear quarter developed only transitory clinical signs, but the CMT reactions were strongly positive. Isolations of *N. asteroides* were made only from the right front quarter. Subsequent reinoculation of the left rear quarter and primary inoculation of the left front quarter with approximately 100 billion organisms of strain D again did not produced an enduring infection in this cow, and only transitory signs of clinical illness coupled with positive CMT readings were seen.

Fig. 7—Photomicrograph of lung lesion of cow 52. This section was taken through the same lesion shown in Figure 6 and shows the organisms completely filling the capillary spaces and outlining the intervening alveolae. Gram-Weigart stain; x 230.

Following a dry period of 5 months and subsequent parturition, nocardias were not isolated from her milk for 3 weeks postpartum. The cow was killed for necropsy to determine whether the previous infection in the right front quarter had become walled off or had been completely resolved during the intervening dry period. No active foci were encountered. Cultures of the mammary tissue from all quarters and the right supramammary lymph node were negative for Nocardia.

Cow 52.—A mature Guernsey was purchased from the owner of herd E following repeated isolation of *N. asteroides* from her right front quarter. The infection of this gland, in an inapparent state, was originally discovered during cultural examination of the entire herd. The cow passed through the remainder of that lactation and the subsequent dry period without remarkable incident. Within 48 hours of parturition, signs of clinical illness were seen; there was typical high fever and marked clinical mastitis in the right front quarter. *Nocardia asteroides* was cultured from the milk of this gland in the absence of other organisms. The cow was purchased at this point to study the effects of experimental inoculation in an animal already chronically infected with a strain of *N. asteroides* of low virulence.

Six weeks following parturition, the left rear quarter was infused with approximately 10 billion organisms of the strain from herd G. Systemic reaction to this inoculation was meager, and the body temperature did not exceed 102.4 F. However, a persistently progressive infection of the quarter resulted. The quarter became enlarged, hard, and tender within a week of inoculation. Weekly cultural examinations of milk from both the naturally infected and the inoculated quarters were consistently positive for *N. asteroides* during the 15 months this cow was observed.

Fourteen months following inoculation, cow 52 underwent parturition. No dry period was permitted between these 2 lactations. An acute clinical response, including fever, depression, and inappetence, was evident on the 3rd day postpartum. The body temperature reached 108 F. on the 5th day and then dropped until the death of the animal 14 days postpartum. The right front quarter was moderately enlarged following parturition. The left rear quarter was extremely enlarged and was

producing a blood-tinged serous exudate at the time of death.

At necropsy, both the left rear and right front quarters were affected by a diffuse nodular firmness. The cut surface of the left rear quarter had a darkened, cooked appearance and an ammonia-like odor was detected. This quarter appeared to have undergone complete infarction. The linings of the teat sinuses of both infected quarters were thickened. The juncture of the teat sinus with the gland cistern of the right front quarter was partially obliterated by fibrous tissue proliferation.

The supramammary and internal inguinal lymph nodes were enlarged. Necrotic foci were evident along the periphery of the left supramammary lymph node. Adhesions among the liver, reticulum, and diaphragm surrounded an abscess, resulting from traumatic reticulitis. The lungs were diffusely involved with firm, necrotic foci 1 to 2 mm. in diameter. These foci were surrounded by narrow rings of congestion and hemorrhage.

Microscopic examination of tissues from the left rear quarter revealed multiple inflammatory foci with minimal encapsulation. Vasculitis and thrombosis of adjacent vessels were observed.

The histopathologic changes in the right front quarter were similar to those seen in the left rear quarter, with the exception that infarction had not occurred.

Multiple lesions of the lungs were seen closely associated with the pulmonary vessels. These foci consisted of masses of branching mycelian elements surrounded by degenerating neutrophils and a localized area of vascular congestion (Fig. 6). Mycelian emboli were lodged in the capillary bed and outlined entire groups of alveoli (Fig. 7).

Nocardia asteroides was cultured from tissues of all 4 mammary quarters. Milk samples taken from all quarters 5 days before death were also positive for *N. asteroides*. This was the only time the organism was recovered from the noninoculated quarters during the 15-month period of observation. *Nocardia asteroides* was also isolated from the right and left supramammary and left internal inguinal lymph nodes, the lungs, and the spleen. The organism was not recovered from venous blood drawn 1 day before death, or from macerated tissues of the right internal inguinal lymph node.

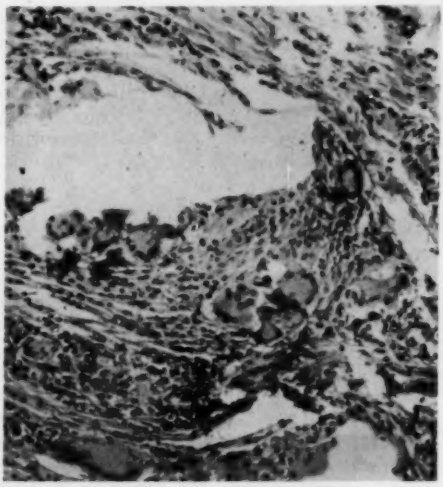

Fig. 8—Photomicrograph showing numerous giant cells seen in lesions of the left rear mammary quarter of cow 328, experimentally infected with Nocardia. H & E stain; x 115.

Cow 328.—A 1st-lactation Ayrshire was inoculated during her 1st week of lactation in the right front and left rear quarters with approximately 10 billion organisms from herds G and E, respectively. Febrile response reached 107 F. by the 2nd day postinoculation. The right front quarter was swollen and hard, and an edematous swelling extended forward to the region of the navel and posteriorly over the right rear quarter. The left rear quarter was not clinically involved. Milk from both quarters reacted strongly to the CMT. The cow became depressed and refused feed on the 4th day, at which time 3 million units of penicillin and 5 Gm. of dihydrostreptomycin were given intramuscularly. Eight days later, the cow was found to be prostrate and had muscular tremors. Dextrose and 500 ml. of 25% solution of sulfamerazine, sulfathiazole, and sulfapyridine were administered intravenously, and 2.0 Gm. of novobiocin was given intramuscularly. Clinical improvement was apparent the following day. The quarters continued to shed *N. asteroides* in the milk following both treatments. Forty-five days following inoculation, a small draining sinus, 2 cm. in diameter, opened on the surface of the left rear quarter from which *N. asteroides* was isolated.

Weekly cultural analyses of the milk of

cow 328 indicated eventual internal distribution of *N. asteroides* to all quarters by the 3rd week of infection. Secondary infection with *C. pyogenes* and coliform bacilli followed the development of sinus tracts, and these organisms also distributed to quarters not directly associated with the sinus tract.

Cow 328 was killed for necropsy 3½ months after experimental infection. The pathologic changes were altered by the presence of *C. pyogenes* and other secondary invaders, but many of the changes seen were typical of udders infected by *N. asteroides* alone.

Both inoculated quarters were extensively fibrosed. The lining of the teat sinus, gland cistern, and the larger collecting ducts were thickened and coarsely granular. In the right front quarter, the parenchyma was mostly replaced by a fibrous mass surrounded by a thick, creamy exudate. The entire lesion was enclosed in a fibrous capsule 1 cm. thick. The parenchyma of the left rear quarter was replaced by fibrous tissue interspersed with abscess cavities up to 4 cm. in diameter.

Microscopic examination of tissue sections revealed extensive inflammatory hyperplasia of the mucosal lining of the teat sinuses of the right front quarter. Encapsulated granulomatous lesions with prominent epithelioid tissue and numerous giant cells were frequently encountered (Fig. 8). The organisms were seen at the inner peripheries of such lesions.

Small, well-encapsulated, granulomatous foci were scattered through the right supramammary lymph node. The left supramammary lymph node and both internal inguinal lymph nodes were edematous but did not contain focal necrotic lesions.

Cow 329.—A 1st-lactation Ayrshire was inoculated in the right front and left rear quarters during her 1st week of lactation with 10 billion organisms from herds D and A, respectively. The rectal temperature reached 105.5 F. by the 2nd day. Four days following inoculation, the left rear quarter was greatly distended and hard; little clinical reaction occurred in the right front quarter, but the milk of both reacted strongly to the CMT. The left rear quarter developed a draining sinus tract 25 days postinoculation (Fig. 3).

Extension of the infection into noninoculated quarters was indicated by repeated isolations of the organism from the left front quarter and right rear quarters by the second week of infection. The rapid

TABLE 5—Response of Cattle Experimentally Infected with Different Isolates of Nocardia asteroides

Cow No.	Qtr.	Isolate	Clinical response		Necropsy	Tissue containing Nocardia
			Glandular	Systemic		
3107	RR	A*	Swelling, exudate, culturally positive.	Severe, treated (temp. 106.6 F.).	Mammary: fibrosis, necrosis, epithelial proliferations; circumscribed, granulomatous lesions.	Mammary gland.
	LF	A*	Swelling, exudate, culturally positive.	.....	Mammary: fibrosis, necrosis, epithelial proliferations; circumscribed, granulomatous lesions.	Mammary gland.
3032	RF	G*	Swelling, exudate, culturally positive (cleared after 3 mo.).	Marked (temp. 105.1 F.).	Mammary: focal fibrosis.	None.
	LR	D**	Transitory, mild, culturally negative.	.....	Normal.	None.
	LF	D**	Transitory, mild, culturally negative.	Mild (temp. 103.7 F.).	Normal.	None.
52	RF	E**	Field infection, culturally positive.	Mild; became marked at 1st parturition (temp. 105.8 F.).	Mammary: extensive fibrosis and necrosis.	Mammary gland, lungs, lymph nodes, liver, spleen.
	LR	G*	Swelling, exudate, culturally positive.	Mild (temp. 102.4 F.); became marked at 2nd parturition (temp.108 F.); terminated fatally.	Thrombosis and infarction. Lesions of lymph nodes and lungs.	Mammary gland, lymph node.

* High virulence. ** Low virulence.

extension of secondary invaders from the left rear quarter to the remaining 3 quarters following exteriorization of the sinus tract indicated the destruction of the anatomical subdivision of the udder.

The necropsy conducted 5 months following inoculation was influenced by the presence of *C. pyogenes* as a secondary invader of the mammary gland and by a traumatic reticulosplenitis.

Inflammatory proliferation of the mucosal lining of the teat sinus of the left rear quarter was seen. The 3 remaining quarters were moderately fibrosed. Nocardias were isolated from the mammary tissues only.

Cow H21.—A purebred Holstein-Friesian in her 4th lactation was inoculated in the right front and left rear mammary quarters with 100 billion organisms from herds F and A, respectively. The body temperature reached 104.6 F. 3 days postinoculation. The milk production dropped precipitously within 24 hours of inoculation. After 48 hours, both inoculated quarters were enlarged and firm and were producing altered secretions; the cow was lacrimating excessively, and a stiff-gaited lameness of the left rear leg was noticeable. By 96 hours, the cow had visibly lost condition, and the swelling of the left rear quarter extended upward into the escutcheon region. Seven days following inoculation, the cow showed signs of depression and was found dead on the morning of the 8th day.

Necropsy of this cow revealed areas of mixed congestion and hemorrhage and small purulent foci in both inoculated quarters. The left supramammary lymph node was enlarged and moist and had a softened, necrotic center. Fibrin tags overlaid the lungs, and rough fibrinous areas were seen on the pleural surfaces of the rib cage. Contained in the pericardium was 250 ml. of serous fluid.

Nocardia asteroides was isolated from the tissues of both inoculated quarters and from the left supramammary lymph node.

Cow 119.—A mature Jersey in midlactation was given 20 billion organisms from herds A and G in the right rear and left front quarters, respectively. A response typical of an acute field case followed. Marked swelling and fibrosis with palpable hard lumps were evident in the right rear quarters by the 4th day postinoculation. At this time, the cow's body temperature was 106 F.; both inoculated quarters were en-

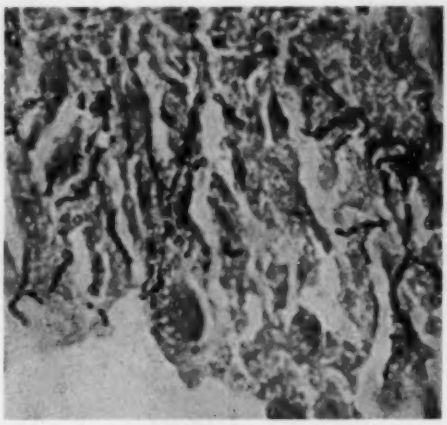

Fig. 9—Photomicrograph showing Nocardia asteroides distributed along the inner edge of a mammary lesion in cow 119, experimentally infected. Gram-Weigart stain; x 800.

Fig. 10—Lung (above) and supramammary lymph node (below) from experimentally infected cow 119. Lung lesions were uniformly distributed throughout the organ, suggesting hematogenous metastasis. Lesions of the lymph node were concentrated opposite the hilus, suggesting distribution via afferent lymphatics.

TABLE 6—Response to Experimental Infection

Cow No.	Qtr.	Isolate	Clinical response: Glandular	Clinical response: Systemic	Necropsy	Tissue containing Nocardia
328	RF	G*	Extensive swelling, exudate; culturally positive.	Severe, treated (temp. 107 F.).	Mammary: extensive necrosis and fibrosis; sinus tract: granulomatous lesions; giant cells; epithelial hyperplasia. Granulomatous foci in lymph node.	Mammary gland.
	LR	E**	Transitory, mild, culturally positive.	.....	.....	.....
329	RF	D**	Transitory, mild, rarely shed organism.	Severe (temp. 105.5 F.).	Mammary: extensive fibrosis, necrosis and granulomatous foci.	Mammary gland.
	LR	A*	Extensive swelling, exudate; culturally positive.	.....	.....	.....
H21	RF	F*	Swelling, exudate, culturally positive.	Severe, fatal (temp. 104.6 F.).	Mammary: focal necrosis, thrombosis, hemorrhage.	Mammary gland, lymph nodes.
	LR	A*	Swelling, exudate, culturally positive.	.....	Lesions of supramammary node.	.....
119	RR	A*	Swelling, exudate, culturally positive.	Severe, fatal (temp. 106.6 F.).	Mammary: focal lesions, hemorrhage, epithelial proliferation.	Mammary gland, lymph nodes.
	LF	G*	Swelling, exudate, culturally positive.	.....	Focal lesions, lymph node and lungs.	Lungs.

* High virulence. ** Low virulence.

larged and tender, and there were fibrinous clots in the milk. The body temperature reached 106.6 F. on the 5th day. The milk from the inoculated quarters became blood-tinged on the 10th day. Following a course of febrile reaction, the cow died 12 days after inoculation.

At necropsy, the mammary gland had roughened proliferative areas on the mucosal linings of the teat sinuses and ducts. Massive hemorrhagic and congested areas with necrotic foci were grossly visible on the incised surface. Microscopic examination revealed numerous granulomatous foci. Nocardias were demonstrable along the inner borders of these lesions (Fig. 9).

The lungs and pleural surfaces bore fibrinous tags. Many caseous foci, 2 to 3 mm. in diameter, were seen in both the supramammary lymph nodes and throughout the lungs (Fig. 10). Neither gross nor microscopic lesions were seen in the heart, liver, spleen, or the supramammary lymph nodes. Cultural examinations of these tissues, the heart blood, and the pleural effusate did not reveal *N. asteroides*. The organism was isolated from the tissues of the inoculated mammary quarters and from lesions of the supramammary lymph nodes and the lung.

The temperature response and the results of cultural examination of the milk of each experimentally infected animal are presented (Fig. 11, 12); the results of hematologic observations (Table 4) and a summary of clinical and necropsy findings (Tables 5, 6) are also presented.

DISCUSSION

There was unusual constancy in the cultural reactions of the 7 isolates (Table 1), with the notable exception of the colonial appearance of organisms from herd F as the result of the production of aerial myceliums. The action of the isolates on guinea pigs was less constant and degrees of virulence were apparent. Strains which regularly killed guinea pigs when 1 billion organisms were injected did so in from 4 to 9 days; the remaining strains in general did not kill in the 14-day period. On this basis, it would seem probable that at least 4 of the strains possessed unusual virulence.

The results of these guinea pig reactions coincide with the nature of the clinical disease seen in naturally infected animals and correlate with the morbidity rates in the infected herds. It is felt that management practices also contribute to the latter figure.

Studies on experimentally infected cattle have been used to further substantiate this virulence gradient. In cows 3032, 52, 328,

and 329, the strains inoculated into separate quarters differed markedly in their virulence for guinea pigs (Table 2). In each case, the severity of clinical response clearly marked the quarter inoculated with the strain of high virulence.

In cow 3032, only the more virulent strain produced a continuing infection, and this cleared spontaneously during a prolonged dry period. Attempts to infect a 3rd quarter with the less virulent herd D isolate again did not result in detectable infection. An organism combination of similar virulence differential was imposed on cow 52. This cow was already naturally infected with a strain of low virulence. An enduring infection of the quarter inoculated with the strain of high virulence resulted.

In cows 3107, H21, and 119, 2 quarters of each cow were inoculated with organisms of high virulence. Two of these cases terminated fatally with culturally demonstrated infection of both inoculated glands and culturally positive foci beyond the mammary gland proper (e.g., supramammary nodes of cows H21 and 119 and the lungs of cow 119). Cow 3107, also infected in 2 quarters with the highly virulent strain from herd A, reacted in such a severe manner that a fatality was feared and therapy was imposed on the clinical course of infection. The only other fatality resulting from experimental infection occurred in cow 52. This cow had survived an earlier parturition while infected in 1 quarter with a strain of *N. asteroides* of low virulence. A later parturition, following experimental infection with a strain of high virulence and complicated by traumatic reticulitis, resulted in wide-spread dissemination of the nocardial infection and death of the animal.

The distribution of the lesions along the periphery of the supramammary lymph nodes in cows 52, H21, and 119 indicated

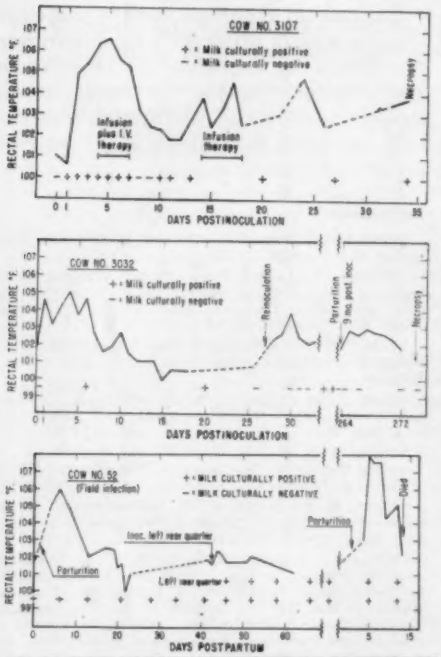

Fig. 11—The temperature response of cows following experimental inoculation with Nocardia asteroides.

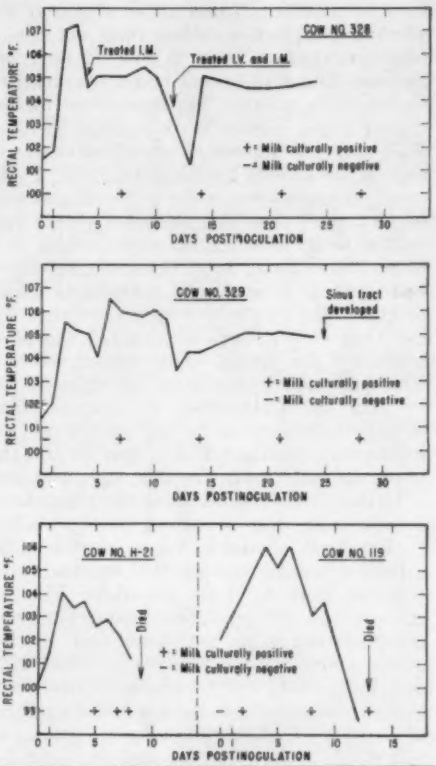

Fig. 12—The temperature responses of cows following experimental inoculation with Nocardia asteroides.

that metastasis to these nodes occurred via afferent lymphatics rather than via hematogenous routes. Only in cow 52 were organisms found in lymph nodes proximal to the supramammary. The absence of foci in lymph nodes higher in this chain in cows H21 and 119 indicated an effective clearance of the stream by the distal node. Pathologic changes seen in the infected quarters of all 3 cows that died included severe vasculitis with necrosis of vessel walls and thrombosis. In all fatal cases, the infection had spread beyond the mammary gland proper. The route by which the lungs became infected in cows 52 and 119 was most probably the result of a venous erosion which allowed a shower of infective emboli to gain the pulmonary circulation. The demonstration of infective emboli in the pulmonary capillary bed of cow 52 and the apparent uniformity in size, age, and distribution of the lesions along the pulmonary vessels make this sequence most probable.

Histologic changes from experimental infection seemed less affected by strain differences than by time duration. The reactions varied from diffuse hemorrhage and consolidation with purulent foci in the acute, short-lived infections of cows H21 and 119 to only the beginning of proliferative reactions seen on the epithelial surfaces of cow 119, 12 days following infection. In cow 3107, whose quarters were infected for 1 month, a greater degree of encapsulation of lesions was evident and the proliferations of epithelial surfaces were more marked. Lesions seen after infection of 3 months' duration in cow 328 were clearly granulomatous in nature. Hyperplastic changes of the epithelial surfaces of the teats and lower ducts of cow 328 were evident, but these findings were shadowed by the presence of secondary invaders, as were the necropsy findings in cow 329.

Periodic hematologic observations made during the course of these infections reveal a progressive increase in the percentage of neutrophilic leukocytes, with a shift toward the precursors of the polymorphonuclear leukocytes. The total leukocyte count exceeded normal ranges in only 1 instance, when a count of 22,450/cmm. was observed during the 2nd week of infection in cow 119. A progressive lymphocytopenia was observed early in the course of infection in cows 3107, 328, and 119. The course of infection in each of these 3 cows was severe and terminated fatally in cow 119.

SUMMARY

Nocardia asteroides was demonstrated as the etiologic agent of a severe and persistent mammary infection of cattle in 7 dairy herds in California and 3 in Hawaii.

The organisms isolated from 7 of the herds were shown to have constant mycologic features but varied with respect to their virulence for guinea pigs.

The clinical disease in naturally infected cattle usually closely followed parturition of multiparous individuals. The disease varied in severity, which corresponded to the variable virulence of the isolates for guinea pigs.

The more severe form of the disease was denoted by high body temperature and a rapidly progressive fibrosis of the infected glands, often accompanied by the development of sinus tracts or rupture of the gland. Granulomatous lesions were seen in infected mammary tissue. Metastasis to the lungs and supramammary lymph nodes was observed. The less severe form of the disease was usually relegated to progressive fibrosis of the infected mammary quarters.

The clinical syndrome and the pathologic changes seen in cattle with field cases were reproduced by experimental inoculations. The clinical response following experimental inoculation uniformly coincided with variations in virulence of the organism for guinea pigs.

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SUMMARIO IN INTERLINGUA

Nocardia asteroides Como Pathogeno Mammari in Animales Bovin. I. Le Morbo in Bestial e le Virulentia Comparative de Cinque Isolatos

Nocardia asteroides esseva demonstrate como le agente etiologic de un sever e persistente infection mammari de bestial in 6 greges lactific de California e 3 de Hawai.

In le caso de 7 del greges, il esseva monstrate que le organismos isolate habeva constante characteristicas mycologic sed que illos variava con respecto a lor virulentia in porcos de India.

In naturalmente inficite bestial, le morbo clinic usualmente sequeva le parturition de individuos multipare post breve intervallos. Le morbo variava in severitate, in correspondentia con le virulentia variabile del isolatos in porcos de India.

Le forma plus sever del morbo esseva characterisate per alte temperaturas corporee e un rapidemente progressive fibrosis del inficite glandulas, frequentemente accompaniate per le disveloppamento de tractos de sino o ruptura del glandula. Lesiones granulomatose esseva vidite in le inficite tissu mammari. Metastase al pulmones e al nodos lymphatic supramammari esseva observate. Le forma minus sever del morbo esseva usualmente relegate al progressive fibrosis del inficite quartieros mammari.

Le syndrome clinic e le alterationes pathologic vidite in bestial a infection in le campo esseva reproducite per inoculationes experimental. Le responsa clinic post inoculation experimental coincideva uniformemente con variationes in le virulentia del organismo pro porcos de India.

Foot-and-Mouth Disease Virus. I. Propagation of 3 Immunologic Types of Virus in Chicks

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MOST of the previous studies on the propagation of foot-and-mouth disease (FMD) virus in chicks were limited to newly hatched chicks.

The susceptibility of older birds to infection with FMD virus was described by Skinner.[15,16] After inoculating the tongue epithelium with either a bovine or a guinea pig strain of virus, it was found that local lesions usually developed at the site of inoculation. In this way, the virus was transmitted successfully for 6 serial passages in birds 2 weeks to 4 months old.

In previous work,[19] the influence of age on the multiplication of FMD virus as well as on the development of pathologic lesions in infected chicks, 1 and 9 days old, was investigated. Although the smallest dose of virus required for infecting day-old chicks was about 10 times less than that required to infect 9-day-old chicks, the day-old chicks developed a higher virus concentration and more severe gizzard lesions. It was suggested, therefore, that day-old chicks were more susceptible than older ones.

Recently, the possibility of adapting FMD virus to adult rodents by the use of animals of gradually increasing ages has been described by several workers.[2,17,18] By this method, some modified strains of virus of immunizing value have been obtained.

This report contains the results of studies on the propagation of FMD virus of types Vallée A and O and Waldmann C in chicks 1 day old as well as in chicks 1, 2, 3, and 4 weeks old.

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From the Pan American Foot-and-Mouth Disease Center, Rio de Janeiro, Brazil (a project of the Pan American Sanitary Bureau and financed by the Organization of American States). Dr. Zahran is now with the Veterinary Research Laboratories, Ministry of Agriculture, Dokki, Cairo, Egypt, U.A.R.

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MATERIALS AND METHODS

Virus Type Vallée A (Strain Cruzeiro).—A Brazilian strain of bovine origin, transmitted to day-old chicks for 13 passages,* produced generalized infection in 4 cattle inoculated intradermally in the tongue. This strain was available in the form of a gizzard tissue sample stored at −20 C. for 442 days.

Virus Type Vallée O (Strain Lara).—The Vallée O type of FMD virus was a Venezuelan strain of bovine origin. After 1 passage in day-old chicks and 2 successive passages in unweaned mice, it had been passaged serially in day-old chicks up to the 45th passage.[12] When this strain was tested in cattle at the 44th chick passage, it produced lesions on the tongues, lips, and gums of 2 susceptible calves.

This virus was available in the form of a 10% pooled heart and gizzard muscle suspension stored at −20 C. for 667 days. The titer of this sample was $10^{2.5}$ mouse LD_{50} per 0.05 ml.

Virus Type Waldmann C (Strain Rezende).—The Waldmann C type, a Brazilian strain of bovine origin, had been maintained for 30 serial passages in tissue culture of bovine tongue epithelium according to Frenkel's method. The titer of a sample from this virus strain was 10^7 mouse LD_{50} per 0.05 ml. It was obtained in the form of bovine tongue epithelial tissue which had been kept at −20 C. for 35 days.

Passaging of Virus in Chicks.—Day-old New Hampshire Red chicks were obtained twice weekly from a poultry farm. Older chicks used were kept in the laboratory until required. Three to 14 chicks were inoculated for each passage. Occasionally, 2 to 5 chicks, not inoculated, were kept as controls. A dose of 0.5 to 1.0 ml. of a 10 to 20% suspension of gizzard or heart, or both, prepared with phosphate buffer (pH 7.6) and clarified by centrifugation, was inoculated intramuscularly in the thigh. Such suspensions were treated with 1,000 I.U. of penicillin, 100 μg. of streptomycin, and 100 I.U. of nystatin* per milliliter.

Chicks, either affected with FMD or normal, were killed with chloroform 2 to 4 days after inoculation. Their carcasses were immersed in a weak saponified solution of cresol. Dead chicks were preserved at −20 C. and disinfected similarly after thawing. Necropsies were carried out with a mini-

* Dr. Rafael Fuentes Marins, Instituto de Investigaciónes Veterinarias, Maracay, Venezuela.

* Mycostatin, produced by E. R. Squibb & Sons, New York, N.Y.

TABLE 1—Incidence of Lesions and Deaths Among Chicks Infected with Foot-and-Mouth Disease Virus

	Virus A					Virus O					Virus C				
Age of chicks (days)	No. of chicks	Lesions (%)			Deaths (%)	Age of chicks (days)	No. of chicks	Lesions (%)			Deaths (%)	Age of chicks (days)	No. of chicks	Lesions (%)			Deaths (%)
		G	H	T				G	H	T				G	H	T	
1	120	87	20	5	0	1	132	77	93	22	76	1	151	93	43	34	5
6–8	77	74	23	0	0	6–8	42	73	100	0	50	6–8	49	98	71	0	20
14–15	42	93	24	0	0	14–15	29	75	100	0	58	14–15	54	98	77	2	26
21–22	18	88	11	0	0	21–22	88	70	73	1	18	21–22	51	88	80	4	20
28–29	....	....	....	..	..	28–29	26	65	84	0	27	28–29	18	83	72	0	0
Total and mean	257	85.5	19.5	1.25	0		317	72	90	4.6	45		323	92	68	8	14

G = gizzard; H = heart; T = tongue.

mum of delay, and specimens from the gizzard and heart were collected aseptically for further transfers. Occasionally, other tissues were also collected for quantitative viral assay.

Bacteriologic sterility tests were carried out in order to provide a bacteria-free seed virus. Macroscopic lesions in the gizzard, heart, and tongue were recorded, and they were evaluated according to the score method described in a previous paper.[19]

Titration of Virus.—Unweaned mice, 6 to 9 days old, were used according to Skinner's method[14]; 0.05 ml. of serial tenfold dilutions of suspended tissues was inoculated intraperitoneally, using 4 to 8 mice for each dilution. The inoculated mice were kept under observation for 8 days. Virus titers were expressed as the number of mouse LD_{50} in 0.05 ml. of the undiluted material, calculated according to the method of Reed and Muench.[18]

Typing of Virus.—Either the infected gizzard tissue of chicks or the muscle tissue of mice inoculated with the chick-propagated virus were tested for their type specificity by the complement-fixation test.

Experimental Animals.—Crossbred cattle, 1 to 2 years old, with Zebu characteristics predominating, were obtained in the State of Rio de Janeiro, Brazil. They were inoculated intradermally either in the tongue epithelium according to Henderson's method[8] or in the skin of the scapular region. Observations were continued for 15 days, and serums were collected before as well as 1 and 2 weeks after inoculation. The immunity of inoculated cattle and 1 or 2 control animals was challenged by intradermal inoculation of the tongue with bovine virus; each animal was given 4×10^5 mouse LD_{50}.

Guinea pigs were inoculated intradermally in the left metatarsal pad. They were kept under observation for 10 to 14 days. Occasionally, the immunity of inoculated animals was challenged 1 to 2 months later, with guinea pig adapted virus. Two susceptible guinea pigs were infected similarly to serve as challenge controls.

Serum Protection Test.—Serums collected from cattle were examined for the corresponding antibodies by serum protection tests in unweaned mice. The procedure adopted was that described by Cunha *et al.*[1]

Results

Passaging of Virus in Chicks

Virus Type A, Strain Cruzeiro.—The type A virus was passaged in 4 series of chicks of gradually increasing ages. Virus was transferred up to the 25th passage in day-old chicks, to the 40th passage in 7- and 8-day-old chicks, and to the 50th passage in 14- and 15-day-old chicks. Five more passages were performed in 21- and 22-day-old chicks. In most of these transfers, the virus was readily propagated in the older chicks used, except the 35th passage which did not infect 2-week-old birds.

Virus Type O, Strain Lara.—Type O virus was passaged in 5 series of chicks of gradually increasing ages. It was transferred up to the 50th passage in day-old chicks, and to the 55 passage in 6- and 7-day-old chicks. The 56th passage only was inoculated into 11-day-old birds. Further passaging was carried out from the 57th to the 61st passage in 14- and 15-day-old chicks and up to the 77th passage in 21- and 22-day old chicks. From the 78th to the 84th passage, virus was transferred in 28- and 29-day-old birds. Chicks of all ages used were found readily susceptible to this strain of virus,

Virus Type C, Strain Rezende.—Type C virus was transferred for 15 serial passages in day-old chicks. Afterward, birds 7 and 8 days old were used to the 25th passage, and 14- and 15-day-old birds were used until the 35th chick passage. Propagation of virus was then continued to the 45th passage in 21- and 22-day-old chicks. Five more passages were carried out in chicks 28 and 29 days old. Chicks 1 to 29 days old were found to be readily susceptible to infection with this strain of virus.

Pathologic Changes.—The 3 virus strains used in this study produced different pathologic changes in infected chicks.

In 257 chicks infected with virus type A, neither clinical signs nor deaths were observed. Necropsies performed on these chicks revealed, however, the characteristic gizzard and heart lesions described by Gillespie [6,7] and confirmed [19] to be specific for FMD virus infection.

Tongue lesions were noticed on only 7 occasions in day-old chicks and were never seen in later passages in older birds (Table 1, Fig. 1).

Of 257 chicks, 1 to 22 days old and infected with the Cruzeiro strain of virus type A, 1.25% developed tongue lesions, 85.5% developed gizzard lesions, and 19.5% developed heart lesions.

Two to 3 days after inoculation with either virus type O or C, some chicks were inappetent and drowsy. A few hours to 1 day later, they died without having shown serious signs. The rate of mortality among infected chicks was usually more frequent in the higher chick passage levels of various groups. It was more evident in day-old chicks infected with virus type O than in older chicks. Upon necropsy, sick or dead chicks usually were seen to have yellowish serous fluid in the abdominal cavity and pericardial sac, as well as congestion in the internal organs, especially in the liver and kidney.

Heart lesions occurred most frequently in chicks infected with virus type O (Table 1, Fig. 2). Gizzard lesions were also noticed in a high percentage, up to a mean of 72%. Tongue lesions were noticed in 22% of day-old chicks and in 1% of 3-week-old birds infected with virus type O.

Gizzard lesions, noticed in 92% of all chicks, were found most frequently in chicks infected with virus type C (Table 1, Fig. 3). Lesions in the heart were observed in 68% of the same birds. Tongue lesions, evident in 34% of the day-old chicks, decreased to 2 and 4% in the 2- and 3-week-old birds, respectively.

Three chicks, 21 days old, were infected with the 54th chick passage of virus A, and 3 chicks, 28 days old, were infected with the 83rd chick passage of virus O. Chicks of both groups were inoculated intradermally in the tongue epithelium in 2 to 3 sites

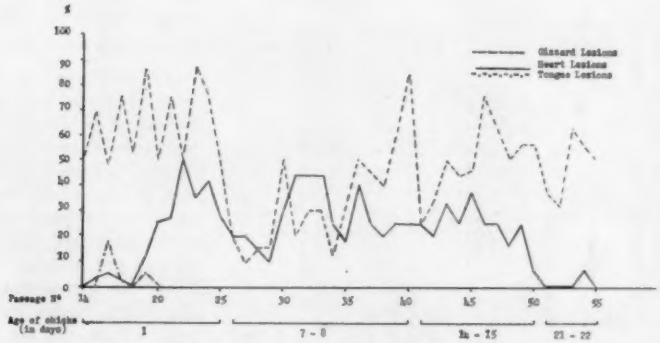

Fig. 1—Lesions in chicks infected with foot-and-mouth disease virus type Vallée A.

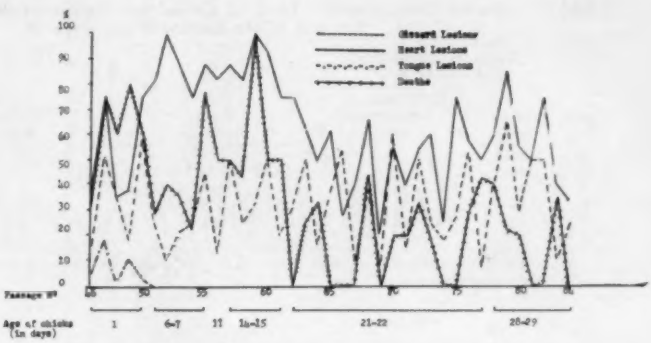

Fig. 2—Lesions in chicks infected with foot-and-mouth disease virus type Vallée O.

with a 20% gizzard suspension of the corresponding virus passage. Tongue lesions were not found in any chick. Titration end points of suspensions of gizzard and tongue from chicks infected with the 54th passage of virus type A were $>10^{4.5}$ and $<10^{1.0}$ mouse LD_{50}, respectively.

In a similar experiment with the 41st chick passage of virus type C, 2 of 4 chicks, 21 days old, developed tongue lesions at the site of inoculation.

The percentage lesion score in the gizzards, hearts, and tongues of chicks of different ages was plotted (Fig. 1–3). Day-old chicks usually had the most developed lesions. When the passaged virus was transmitted from one series of chicks to another of older chicks, development of lesions decreased. They become more evident in the gizzard and sometimes in the heart after progressive passaging of virus in older chicks.

Specificity of Deaths in Infected Chicks.—To determine the specific cause of death among the chicks infected with the type O strain of virus, serum neutralization tests were performed in day-old chicks and in unweaned mice.

A gizzard suspension of the 47th chick passage of virus type O was titrated in day-old chicks. The titer obtained was $10^{6.4}$ LD_{50} per 1.0 ml. The same suspension was also titrated in unweaned mice and the titer found was $10^{7.5}$ per 0.05 ml. Two preparations, one containing 400 chick LD_{50} per 0.5 ml. and the other containing 400 mouse LD_{50} per 0.025 ml., were mixed with dilutions of inactivated type O immune cattle serum. Virus controls were also prepared by mixing similar doses of virus with inactivated normal cattle serum diluted 1 to 5. All mixtures were incubated for 1 hour at 25 C. and then inoculated into groups of 5 chicks (1 ml. per chick, intramuscularly) or into groups of 8 mice (0.05 ml. per mouse intraperitoneally). The results obtained are shown (Table 2).

There were many deaths among the chicks and mice inoculated with mixtures of virus and high dilutions of immune serum or virus and normal cattle serum; the complement-fixation tests carried out with the tissue of dead mice were positive to the type of virus inoculated. However, all chicks and mice inoculated with mixtures of virus and lower dilutions of immune serum were protected against the lethal effect of the virus. These results confirmed the previous finding that the deaths observed in infected chicks were specifically due to the effect of the FMD virus.[19]

Concentration of FMD Virus in the Various Tissues of Chicks.—Samples from various tissues of 4 to 6 chicks used for different passages of virus types A, O, or C were collected. The viral contents of these samples were titrated in unweaned mice. Usually, infected chicks had been inoculated intramuscularly with 1.0 ml. of 10 to 20% gizzard suspension, then were killed 2 to 3 days after inoculation.

The results obtained from the infected gizzards, hearts, and tongues are shown (Fig. 4–6). Results with other tissues, such as the lung, liver, spleen, kidney, pancreas, intestine, skeletal muscle, brain, bone, skin, blood, and bile, are given (Table 3).

The results of these titrations showed that all samples from 1- to 7-day-old chicks infected with either virus type A, O, or C contained variable amounts of virus. Generally, the greatest concentration of virus was found in the gizzard.

A marked decrease to more than 2 logs

TABLE 2—Serum Neutralization Tests in Chicks and Unweaned Mice with Chick-Adapted Foot-and-Mouth Disease Virus Type O

Group No.	Inoculum in chicks and unweaned mice: Virus LD_{50}	+	Dilution of immune cattle serum	Deaths in chicks	Lesions in chicks: Gizzard	Heart	Tongue	Deaths in mice
1	400		1:1	0/5	.....	.....	.....	0/8
2	400		1:10	0/5	.....	.....	.....	0/8
3	400		1:100	0/5	.....	.....	.....	0/8
4	400		1:1,000	0/5	.....	.....	.....	0/8
5	400		1:10,000	5/5	0/5	4/5	0/5	5/8*
6	400		1:5**	5/5	0/5	5/5	1/5	8/8

* Complement-fixation test of dead mice tissue was positive to foot-and-mouth disease virus type O.
** Normal cattle serum.

in virus titer was noticed in the various tissues (except the gizzard and, occasionally, the heart) when that virus was transmitted to older chicks.

Paraf *et al.*[10,11] and Verge *et al.*[18] reported similar observations during the adaptation of FMD virus type C in rabbits of increasing age. The virus was uniformly present in all organs tested (heart, skeletal muscles, brain, and liver) in rabbits less than 8 days old. In rabbits more than 15 days old, the heart and skeletal muscles, the tissues of particular localization of virus in this animal, were found to contain 10^9 mouse LD_{50} of virus per gram; whereas, brain and liver had much lower titers of virus 10^4 to 10^5 mouse LD_{50} per gram.

The virus concentration in the tongue, gizzard, and heart coincided to some extent with the incidence of pathologic changes in the corresponding organ. For example, in early chick passages of virus type C, where lesions in the tongue were evident in 34% of the infected chicks, the virus content of the tongue was much higher than in later pasages, when the lesions occurred in only 2 to 4% of birds. In chicks infected with virus type A, gizzard lesions were most frequently noticed, and the tissues of that organ contained the highest concentration of virus. In chicks infected with virus type O, where little difference was noticed between the incidence of lesions in gizzards and hearts, both organs contained similar viral concentrations.

Virus Multiplication Curve in Chicks 1 and 28 Days Old.—Chicks of 2 groups, 1 day old and 28 days old, respectively, were inoculated intramuscularly with 1 ml. of 10% gizzard suspensions of virus type O prepared from the 50th and 80th chick passages, respectively. The first group contained 21 chicks; the other, 10 chicks.

Due to the occurrence of many deaths in the first group, 3 chicks were killed on post-inoculation days 1, 2, and 5; 2 on day 3 and 1 on day 4. In the second group, 2 chicks were killed daily.

Gizzard tissue was collected from chicks of both groups and titrated in unweaned mice (Table 4).

TABLE 3—Virus Concentration in the Various Tissues of Chicks Infected with Foot-and-Mouth Disease Virus

Chick passage	Age of chicks (days)	Tissues Tested: Gizzard	Heart	Blood	Lung	Liver	Kidney	Spleen	Pancreas	Intestine	Bile	Bone	Tongue	Skel. musc.	Skin	Brain
VIRUS TYPE A																
25	1	6.0	4.3	3.6	3.9	3.7	3.7	3.6	n.t.	2.7	3.1	4.2	3.5	2.6	n.t.	3.0
50	14	5.5	1.4	1.5	1.2	2.3	<1.0	3.2	2.0	3.5	0.5	<1.0	<1.0	<1.0	3.4	<1.0
VIRUS TYPE O																
50	1	6.9	6.7	5.6	4.6	4.6	5.0	3.7	n.t.	4.3	3.5	4.8	4.0	3.8	n.t.	2.6
75	21	5.7	4.8	4.3	3.5	4.5	3.5	2.7	n.t.	3.7	1.9	2.5	3.5	<1.0	2.7	n.t.
VIRUS TYPE C																
25	7	5.1	5.0	4.5	4.2	3.6	3.0	3.2	n.t.	2.3	3.5	3.5	2.5	1.0	n.t.	1.5
45	21	5.0	2.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	1.0	<1.0	<1.0	1.0	<1.0	1.5	<1.0

Virus titer is expressed as logarithm number of mouse LD_{50} per 0.05 Gm. of tested material.
* n.t. = not tested.

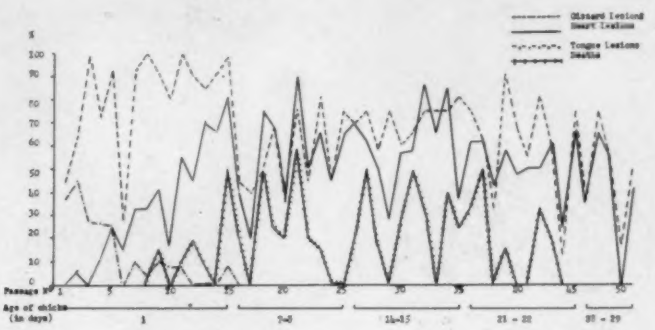

Fig. 3—Lesions in chicks infected with foot-and-mouth disease virus type Waldmann C.

In both groups of infected chicks, the virus was demonstrated from the 1st to the 5th day after infection, *i.e.*, the duration of the period of test. The highest concentration of virus was obtained on day 3 and the lowest on day 5. Gizzards of chicks 28 days old contained, from postinoculation days 2 to 5, about 1 log mouse LD_{50} of virus less than those of day-old chicks. This was accompanied, in older chicks, by fewer pathologic lesions in the gizzard and heart tissues.

Pathogenicity of the Chick-Adapted Virus in Guinea Pigs.—Virus suspensions from gizzards or hearts, or from a pool of both, were prepared from several chick passages of virus type A, O, and C. Ten to 20% dilutions of these suspensions were inoculated into the metatarsal pads of guinea pigs. Of 33 guinea pigs inoculated with different chick-passage levels of virus A, 7 were negative, 15 developed a local reaction, and 11 developed signs of generalized infection. However, 22 of 23 and 14 of 19 guinea pigs inoculated with chick passages of virus O and C, respectively, developed signs of generalized reaction.

The immunity of a number of these guinea pigs was later challenged by inoculation with guinea pig strains of virus. The results were equivocal, as a few that had developed local lesions to the chick virus were not immune; whereas, a few that had not reacted were immune.

Pathogenicity of the Chick-Adapted Virus in Cattle.—Gizzard or heart suspensions prepared from the 50th, 70th, and 45th chick passages of virus types A, O, and C,

TABLE 4—Virus Multiplication in Gizzard Muscle of 1- and 28-Day-Old Chicks Infected with Foot-and-Mouth Disease Virus Type O, and the Occurrence of Heart and Tongue Lesions

Days after inoculation	One-day-old chicks infected with the 50th passage virus						28-day-old chicks infected with the 80th passage virus					
	No. of deaths	No. of chicks killed	Lesions* G	Lesions* H	Lesions* T	Log mouse ID_{50}/Gm. gizzard	No. of deaths	No. of chicks killed	Lesions G	Lesions H	Lesions T	Log mouse ID_{50}/Gm. gizzard
1	0	3	+	++	—	3.9	0	2	—	—	—	4.5
			+	++	—				—	—	—	
			—	—	—							
2	3	3	++	++++	—	6.6	0	2	—	++	—	5.2
			++	++++	—				—	++	—	
			++	++++	—							
3	5	2	++	++++	—	6.8	0	2	+++	++++	—	5.8
			—	++++	—				+	—	—	
4	1	1	+++	+++	—	4.3	0	2	+++	++	—	3.3
									+	—	—	
5	0	3	++	+	—	2.7	0	2	+	++	—	1.5
			+	—	—				—	—	—	
			—	—	—							
Total	9	12	9	9	0		0	10	5	5	0	....

The gizzards from dead chicks were not included in the titration of virus in the muscle.
* G = gizzard; H = heart; T = tongue; + to ++++ indicates lesions of increasing severity.

TABLE 5—Results of Inoculation of Susceptible Cattle with the Chick-Propagated Foot-and-Mouth Disease Virus Strains

Virus type	Chick passage No.	Age of chicks (days)	Site of inoculation	Dose of virus	Animal No.	Reaction after inoculation	Serum protection index			Reaction after challenge
							0 day	7 days	14 days	
A	50	15	T	$10^{5.9}$	721	T, 4F	0.0	2.83	>4.66	negative
					722	T	0.57	>4.83	>5.66	negative
O	70	21	T	$10^{4.3}$	643	2F	0.0	2.50	2.26	negative
					644	T, 2F	0.0	>2.26	2.0	negative
				$10^{2.9}$	720	negative	0.0	<1.16	0.0	T, 4F
C	45	21	i.c.	$10^{3.9}$	724	negative	0.50	1.66	0.83	T, 4F
				$10^{4.9}$	725	negative	0.16	2.50	>4.33	negative
A					728					T, 3F
					725					T, 4F
O		Controls for challenge with bovine foot-and-mouth disease virus.			683					T, 4F
					685					T, 4F
C					717					T, 4F

T = tongue; F = feet; i.c. = intracutaneous.

respectively, were inoculated into 7 susceptible cattle. The susceptibility of these cattle was checked by the absence of antibodies as shown by the results of serum-protection tests in unweaned mice.

The routes of inoculation, the reactions of the cattle, the protection indexes of their serums, and the reaction to challenge with the corresponding bovine virus are given (Table 5).

Three cattle inoculated intracutaneously with the 45th chick passage of virus type C did not develop any local or generalized reaction. One of 2 animals inoculated with the 50th chick passage of virus type A developed only a local reaction at the site of inoculation in the tongue, but the other animal developed generalized infections. Both of the animals inoculated on the tongue with the 70th chick passage of virus type O developed generalized infection.

The cattle that reacted to the chick virus were found to be immune to challenge with bovine virus. The 2 nonreactors to $10^{2.9}$ LD_{50} of type C virus were fully susceptible, but the 1 animal given $10^{4.9}$ LD_{50} of this virus type resisted challenge. Five control cattle all developed generalized infections.

DISCUSSION

Successful propagation of FMD virus in newly hatched chicks has been described by several authors. Attempts to adapt the virus to older birds were made by Skinner,[15,16] but for only a limited number of passages.

In this report, a longer series of transfers with virus types Vallée A and O and Waldmann C were performed with chicks of gradually increasing ages (from 1 to 29 days old) for 55, 84, and 50 passages, respectively.

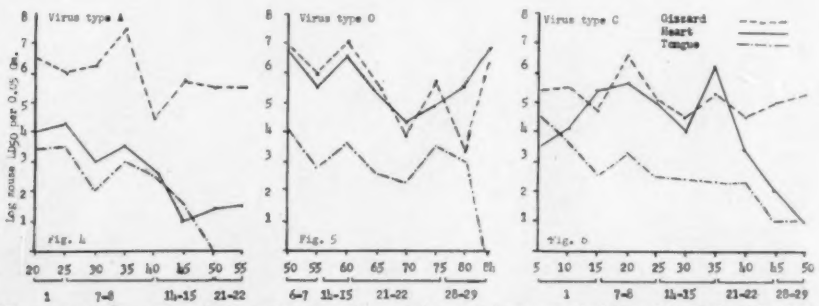

Fig. 4-6—Virus concentrations in gizzards, hearts, and tongues of chicks infected with foot-and-mouth disease virus types A, O, and C. Numbers at the bottoms of the graphs indicate passage number (above) and age, in days, of the chicks (below).

It was hoped that a rapid loss of pathogenicity of virus for cattle would occur. Although virus type C seemed to be well attenuated after 45 passages and virus type A was partially attenuated after 50 passages, virus type O was found to be highly pathogenic for cattle after 70 passages in chicks.

Komarov and Goldsmit [9] found that after 89 and 61 passages of 2 strains of virus types A and O, respectively, in day-old chicks, pathogenicity for cattle was still retained.

In a similar work carried out in rabbits by Cunha and Eichhorn,[2] virus type O was found to be pathogenic at the 96th passage level, but became almost nonpathogenic after the 111th passage. Although virus type C had become somewhat attenuated at the 40th rabbit passage, virus type A was still highly pathogenic at that passage level.

A marked decrease was observed in the virus yield of the various tissues of older chicks, except in the gizzard tissue which proved to contain the highest concentration of virus in the older as well as in day-old chicks.

The practicality of using only infective gizzard tissue as a source of virus for production of a modified virus vaccine is doubtful. However, when the required level of attenuation of the virus for cattle is obtained, it may then be possible to use tissue cultures of susceptible cells in the large-scale production of a modified virus vaccine. The successful cultivation of vaccinia virus in bovine and ovine fetal skin or in explanted tongue epithelium of adult cattle by Frenkel and Kapsenberg [5] and Frenkel [3, 4] are good examples of such investigations.

In considering the possibility of producing a live-virus vaccine from the tissues of chicks or chicken embryos, there is no doubt that the use of chicken embryos would be cheaper and more convenient. The work described in a companion report [20] is of interest in this connection, as a more rapid loss of pathogenicity for cattle and a higher concentration of virus were obtained by passage of the virus in embryonating eggs.

SUMMARY

One Venezuelan and 2 Brazilian strains of foot-and-mouth disease virus, representing types Vallée A, Vallée O, and Waldmann C, were propagated successfully in chicks 1 to 29 days old. The number of passages reached was 84 for virus type O, 55 for virus type A, and 50 for virus type C. Loss of pathogenicity for cattle was observed in 3 cattle infected with the 45th chick passage of virus type C, and in 1 of 2 inoculated with the 50th passage of virus type A.

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SUMMARIO IN INTERLINGUA

Virus de Febre Aphthose. I. Le Propagation de Tres Typos Immunologic del Virus in Gallinettas

Un racia venezuelan e duo racias brasilian de virus de febre aphthose, representante typo Vallée A, typo Vallée O, e typo Waldmann C, esseva propagate a bon successo in gallinettas de etates de inter 1 e 29 dies. Le numero del passages attingeva 84 in le caso del typo O, 55 pro typo A, e 50 pro typo C. Perdita de pathogenicitate pro bestial esseva observate in tres pecias de bestial inficite con le quaranta-quinte passage per gallinettas de virus typo C e in un de duo inficite con le cinquantesime passage de virus typo A.

Foot-and-Mouth Disease Virus. II. Propagation and Modification of 3 Immunologic Types of Virus in Embryonating Chicken Eggs

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TRAUB AND SCHNEIDER [14] reported the first successful transmission of foot-and-mouth disease (FMD) virus to the developing chicken embryo. Peterman,[8] Dedié,[1] Demnitz,[3] Schneider and Schneider,[11] Dedié and Schmidt,[2] and Schmidt [9] used Vallée A and O type strains most often and obtained adaptation only after alternating passages between guinea pigs or mice and the chorioallantoic membranes of eggs incubated for 9 to 11 days.

Federer [4] used a bovine strain of Waldmann type C which was highly pathogenic to cattle. Gillespie [5] succeeded in adapting a strain of the same type of virus in developing chicken embryos after 7 passages in chicks, but did not succeed with similar chick-adapted strains of types A or O.

Using the intravenous route of inoculation, Skinner [12] maintained 4 strains of type O in 14-day embryonating eggs. By applying the same technique, Komarov [6] and Komarov and Goldsmit [7] succeeded in adapting types A, O, C, and Asia I to developing embryos.

In spite of earlier unsatisfactory results, Gillespie,[5] Komarov,[6] Komarov and Goldsmit,[7] and Skinner [13] have recently reported the successful use of a number of modified egg-adapted strains of virus in the immunization of cattle against foot-and-mouth disease.

This report describes the successful propagation and modification of 3 adapted strains of FMD virus (types Vallée A and O, and Waldmann C) in embryonating chicken eggs.

MATERIALS AND METHODS

Source of Virus.—The same strains of FMD virus described in a previous report [15] were used for this study.

Virus Type Vallée A.—Strain Cruzeiro was used at its 35th chick passage; *i.e.*, when it was propagated in 8-day-old chicks.

Virus Type Vallée O.—Strain Lara was at its 50th passage in day-old chicks.

Virus Type Waldmann C.—Strain Rezende was used after being transferred in day-old chicks for 5 passages.

The initial seed virus for each attempt at cultivation in embryonating eggs was contained in a 10% gizzard suspension in phosphate buffer (pH 7.6) that was clarified by centrifugation and treated with 1,000 I.U. of penicillin, 100 μg. of streptomycin, and 100 I.U. of nystatin* per milliliter.

Egg Inoculation.—Fertile eggs from New Hampshire Reds were obtained from the same poultry farm supplying the day-old chicks. Eggs were incubated for 14 days at 37.5 C. before being inoculated with virus. The route of inoculation was either the chorioallantoic membrane, or intravenous. By the 1st method, 0.4 to 0.5 ml. of 10 to 20% pooled gizzard and heart suspension with phosphate buffer (pH 7.6) and antibiotics was inoculated. When the intravenous route of inoculation was used, 0.05 to 0.10 ml. of 1% heart suspension with saline solution (pH 7.6) was inoculated. Reincubation of inoculated eggs was at 37.5 C.

Embryos dying within 24 hours of inoculation were discarded. The virus was harvested from the heart, gizzard, and liver of live as well as dead embryos 2 to 3 days after inoculation. Samples of these tissues were either used directly for further passaging or stored at −20 C.

Bacteriologic sterility tests were carried out with the pooled allantoic and amniotic fluids of harvested eggs as well as with the inoculums.

Titration and typing of virus, guinea pig and cattle inoculations, and serum protection tests were performed by the same procedures previously described.[15]

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* Mycostatin, produced by E. R. Squibb & Sons, New York, N.Y.

RESULTS

PASSAGING OF VIRUS IN EMBRYONATING EGGS

Virus Type A, Strain Cruzeiro.—Four attempts were carried out before the Cruzeiro strain was successfully propagated in embryonating eggs.

In the first attempt, the gizzard suspension prepared from the 30th chick passage, containing $10^{6.2}$ mouse LD_{50} of virus per 0.05 ml., was inoculated on the chorioallantois. Four blind passages were carried out, but the 4th passage proved to be free from virus.

In the 2nd attempt, the 35th chick passage, containing $10^{7.4}$ mouse LD_{50} of virus, was used. Again, after 3 serial egg passages via the chorioallantois, no virus could be detected in the inoculated eggs.

In another trial, the chick-passaged virus was transferred in a series of 4 alternating passages between day-old chicks and eggs. Afterward, 4 more passages were continued by serial transfer in eggs alone, but successful results were not obtained. The intravenous route of inoculation was therefore attempted, and the 4th chick passage of the previous series of alternating passages was used. This seed virus proved to contain $10^{4.5}$ mouse LD_{50} per 0.05 ml. in the gizzards of day-old chicks. Each embryo of the new series was then given $10^{2.5}$ mouse LD_{50} intravenously. Thus, propagation of this strain of virus type A was performed successfully in embryonating eggs until the 20th passage (Fig. 1).

Usually, inoculated eggs were reincubated for 3 to 5 days when the chorioallantoic membrane was used. With the intravenous series of inoculations, 3 days was the usual period of reincubation.

Virus Type O, Strain Lara.—The 50th chick passage of this strain of virus was readily propagated in embryonating eggs for 30 serial passages via the chorioallantoic membrane. The initial seed virus had a titer of $10^{6.9}$ mouse LD_{50} per 0.05 ml. Eggs of the 1st passage were reincubated for 5 days, but afterward for only 2 to 3 days after inoculation.

Virus Type C, Strain Rezende.—After 5 passages in day-old chicks, this strain of virus was successfully adapted to embryonating eggs. For 26 passages, transfer was via the chorioallantoic membrane; 4 were then made by intravenous inoculation. Titer of the seed virus for this series was $10^{5.4}$ mouse LD_{50} per 0.05 ml.

In the 1st egg passage, reincubation after inoculation was for 5 days, but in later passages it was usually 2 to 3 days.

PATHOLOGIC CHANGES

During the 1st alternating chick and egg passages carried out with virus type A, inoculated embryos underwent no change

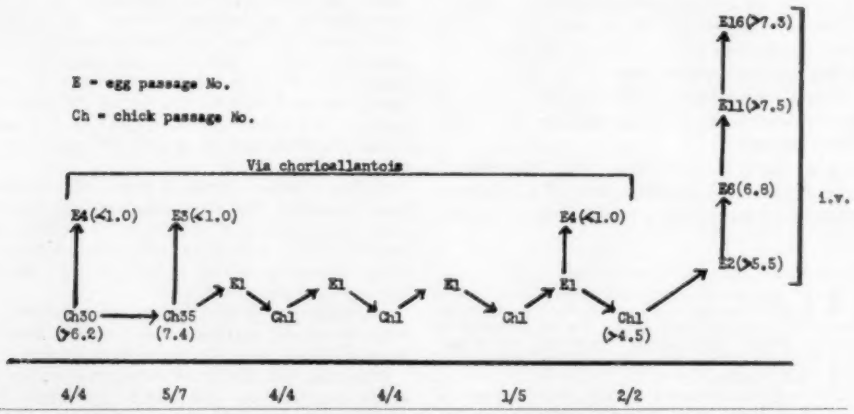

Fig. 1—Direct and alternating passages carried out between chicks and embryonating chicken eggs with foot-and-mouth disease virus type Vallée A. The fractions shown at the bottom of the figure denote the number of chicks with gizzard lesions (numerator) and the number of chicks inoculated (denominator). Numbers shown in parentheses indicate the virus concentration, expressed as log numbers of mouse LD_{50} per 0.05 Gm. of gizzard or heart in chicks and embryos, respectively.

from normal, although the infected day-old chicks used during these passages had definite gizzard lesions (Fig. 1). However, when the virus was injected intravenously, mortality resulted at the 1st passage and increased to more than 90% in high-passage levels. Most of the deaths occurred 50 to 67 hours after inoculation.

Dead embryos had superficial congestion all over the skin, especially on the head, neck, and thorax. The myocardial tissue, in the early passages, contained macroscopic yellowish-white spots similar to those described in 3-week-old chicks. After further passaging of virus, the heart was almost yellowish. Moreover, congestion of the internal organs, especially the liver and kidneys, occurred frequently. In severely affected embryos, abnormal amounts of yellowish serous fluid were found in the abdominal cavity or in the pericardial sac, or both.

Death of embryos also occurred in eggs inoculated with virus type O from the 1st passage onward. The mortality increased to 100% in the high passage levels and was recorded 45 to 67 hours after inoculation. Embryos inoculated with low concentration of virus ($10^{1.7}$ to $10^{2.7}$ mouse LD_{50}) of the 16th egg passage did not die within 3 days after inoculation, although actual multiplication of virus had occurred; $10^{2.0}$ to $10^{6.5}$ mouse LD_{50}, respectively, was found in the heart tissue.

This last observation was also noticed in chicken embryos inoculated with virus type C. Although 66% of the embryos inoculated with $10^{6.3}$ mouse LD_{50} of the 17th passage were found dead 45 to 67 hours after inoculation, yet no deaths occurred among the embryos inoculated with $10^{3.2}$ mouse LD_{50} of the 25th passage within 6 days after inoculation.

Pathologic changes similar to those found in chicken embryos infected with virus type A were also observed in chicken embryos inoculated with either virus types O or C.

CONCENTRATION OF VIRUS IN EMBRYONIC TISSUES

The virus concentration in the various embryonic tissues and fluids of some egg passages infected with either virus type A, O, or C, were detected by titrations performed in unweaned mice (Table 1).

Although variable amounts of virus were found in most of the embryonic materials tested, the highest concentration was usually in heart muscle, liver, and gizzard, in that order. The least amount of virus was found in the allantoic and amniotic fluids and in the yolk sac.

When the chorioallantoic membrane was the site of inoculation for the transfers of virus O, the virus titer of the chorioallantois was $10^{4.5}$ mouse LD_{50}. When the intravenous route of inoculation was used, either during the passage of virus type A or the last 4 passages of virus type C, the chorioallantois did not titer more than $10^{2.7}$ mouse LD_{50} of virus.

Concentration of virus in the embryo, which forms the bulk of the embryonating eggs, varied between $10^{4.5}$ to $10^{5.1}$ mouse LD_{50}.

Titer of virus in lyophilized pools of gizzards and hearts or whole embryos was found to be 1 log of mouse LD_{50} less than in the fresh tissue.

TABLE 1—Virus Concentration in the Various Chicken Embryonic Tissues and Fluids Infected with Foot-and-Mouth Disease Virus

Tissues and fluids titrated	Passage No., virus type A				Passage No., virus type O						Passage No., virus type C					
	6	10	15	20	5	10	17	20	25	30	5	10	17	20	25	30
Heart	....	6.8	>7.5	>7.3	7.5	7.0	>6.5	....	6.7	>7.3	>6.4	5.0	6.3	....	....	5.8
Gizzard	....	3.8	....	4.0	5.5	4.7	....	....	4.2	5.0	3.0	2.7	....	....	....	4.0
Pooled heart and gizzard	>5.5	....	....	....	....	....	....	5.6*	....	....	....	....	....*	....	3.7	....
Liver	....	5.0	3.8	5.0	....	....	....	....	....	....	....	....	....	....	....	4.7
Embryo	....	4.7	....	4.5	....	4.8	....	....	4.7	....	....	5.1	....	4.5**	....	5.0
Chorioallantois	....	2.0	....	<1.0	....	....	....	....	>4.5	....	....	....	....	....	....	2.7
Yolk and yolk sac	....	3.7	....	3.0	....	....	....	....	2.5	....	....	....	....	....	....	3.7
Allantoic and amniotic fluids	....	3.0	....	>3.5	....	....	2.0	....	<1.0	>3.3	....	....	....	....	....	2.0

Virus titer is expressed as log number of mouse LD_{50} per 0.05 Gm. of tested material.
* Titer after lyophilization was $10^{4.7}$. ** Titer after lyophilization was $10^{3.5}$.

TABLE 2—Incidence of Lesions in the Gizzard, Heart, and Tongue of Chicks Inoculated with Egg-Adapted Foot-and-Mouth Disease Virus

Virus type O						Virus type C						Virus type A (Alternating passages)					
No. egg pass.	No. inoc. chicks	No. of chicks with lesions in			No. of dead or sick chicks	No. egg pass.	No. inoc. chicks	No. of chicks with lesions in			No. of dead or sick chicks	No. egg pass.	No. inoc. chicks	No. of chicks with lesions in			No. of dead or sick chicks
		G	H	T				G	H	T				G	H	T	
9	3	2	3	0	1	7	3	3	3	0	1	1	4	4	0	0	0
12	3	3	3	0	3	....	..	..	..	..	..	2	4	4	0	0	0
18	3	3	3	0	2	16	3	3	3	0	3	3	5	1	0	0	0
30	6*	4	6	3**	5	....	..	..	..	..	..	4	2	2	0	0	0
Totals	15	12	15	3	11	....	6	6	6	0	4	..	15	11	0	0	0

G = gizzard; H = heart; T = tongue.
* Three chicks inoculated intramuscularly; 3 inoculated intralingually as well as intramuscularly.
** Inoculated intralingually.

PATHOGENICITY OF THE EGG-ADAPTED VIRUS FOR CHICKS

To find whether the egg-adapted strains of virus types O and C had retained their ability to infect day-old chicks, the following tests were made.

Pooled gizzard and heart suspensions from several egg passages were inoculated into the thighs of day-old chicks, using groups of 3 chicks and a dose of 0.5 to 1.0 ml. On one occasion, 3 chicks were inoculated intralingually as well as intramuscularly. Chicks were killed 3 days after inoculation; the pathologic changes observed are given (Table 2).

The pathologic lesions found in the chicks used during the first 4 alternating passages with virus type A are shown (Table 2) for comparison.

After 16 egg passages of virus type C and 30 egg passages of virus type O, both virus types had retained their ability to infect day-old chicks and to cause pathologic changes similar to those observed before propagating these virus types in embryonating eggs. The 15 chicks inoculated with virus type O developed heart lesions, and 12 of them had gizzard lesions. The chicks inoculated intralingually developed lesions at the site of inoculation. Eleven chicks became sick or died.

Both gizzard and heart lesions were observed in the 6 chicks inoculated with virus type C, and 4 of them were either sick or dead. However, in the chicks infected with the 1st 4 alternating passages of virus type A, there were only gizzard lesions and no deaths were recorded.

PATHOGENICITY OF THE EGG-ADAPTED VIRUS FOR GUINEA PIGS

Ten per cent suspensions of heart, gizzard, and liver and a pool of these tissues were prepared from certain egg passages of virus types A, O, and C. Four or 5 guinea pigs were inoculated with these suspensions intradermally in the left metatarsal pads (Table 3).

Of 18 guinea pigs inoculated with either the 10th egg passage of virus type A, the 10th or the 20th egg passages of virus type O, or the 18th passage of virus type C, 14 developed a generalized infection.

In later passages, the egg-propagated virus types had less tendency to produce a generalized infection. Of 15 guinea pigs inoculated with the 20th passage of virus type A or the 30th passages of virus types O or C, only 4 developed generalized lesions.

PATHOGENICITY OF THE EGG-PROPAGATED VIRUS FOR CATTLE

Suspensions from the hearts (or from a pool of gizzards, hearts, and livers) of chicken embryos infected with the 20th, 24th, and 28th egg passages of virus types A, O, and C, respectively, were inoculated into 6 susceptible cattle, 1 to 1½ years old.

The susceptibility of these cattle was checked by serum protection tests carried out in unweaned mice.

Inoculations were made either subcutaneously, intradermally in the tongue, or in the skin of the scapular region.

The virus suspensions were titrated in unweaned mice with minimum delay after inoculation of the cattle. Fifteen to 21 days later, the immunity of all inoculated cattle as well as that of 5 susceptible control cattle was challenged with the bovine virus.

The reaction in cattle, the serum-protection indexes before and after inoculation with the egg-adapted virus types and the results of challenge with the corresponding bovine virus are shown (Table 4).

TABLE 3—The Reaction of Guinea Pigs Inoculated with Egg-Adapted Foot-and-Mouth Disease Virus

Virus type A					Virus type O					Virus type C				
Egg passage No.	No. inoculated guinea pigs	Reaction after inoculation			Egg passage No.	No. inoculated guinea pigs	Reaction after inoculation			Egg passage No.	No. inoculated guinea pigs	Reaction after inoculation		
		neg.	local	gen.			neg.	local	gen.			neg.	local	gen.
10	5	1	2	2	10	5	0	1	4	18	4	0	0	4
					20	4	0	0	4					
20	5	2	2	1	30	5	0	5	0	30	5	1	1	3
Totals	10	3	4	3	....	14	0	6	8	....	9	1	1	7

Of the 6 cattle inoculated with the egg-adapted viruses, 5 did not develop either a local or a generalized reaction. Only animal 653, inoculated with the 24th egg passage of virus type O, developed a local reaction at the site of inoculation in the tongue.

Serum-protection indexes of 2.3 to 7.8 were obtained in 5 cattle 7 to 21 days after inoculation with the egg-adapted virus types. Only animal 718, inoculated subcutaneously with the 28th egg passage of virus type C, had a low serum-protection index.

These serum test results correspond well with the results of challenge with the bovine virus types. All the cattle that had serum-protection indexes of more than 2.0 proved to be immune. The 1 animal (718) that had an index of less than 2.0 was fully susceptible.

The 5 control cattle used as controls all developed signs of generalized infection.

DISCUSSION

Several authors have attributed their success in propagating FMD virus in embryonating eggs to 1 or more of the following methods:

(1) The use of alternating passages between the animal host in which the virus had been adapted and the chorioallantoic membrane of the chicken embryo; (2) the preliminary adaptation of the virus to chicks; or (3) the use of the intravenous route of inoculation with subsequent incubation of the eggs at slightly lower than usual incubation temperature.

In the present studies, FMD virus type A, which had been maintained in chicks for 30 to 35 passages and then transmitted to embryonating eggs and again to chicks for 4 alternating passages, was successfully propagated in the chicken embryo, but only when the intravenous route of inoculation was used. However, 2 other FMD virus types, O and C, which had been passaged in chicks for 50 and 5 passages, respectively, were readily adapted to embryonating eggs via the chorioallantoic membrane. Both strains had produced in day-old chicks more frequent pathologic lesions and higher virus concentration in the heart than did virus type A. Moreover, they retained their ability to develop the same pathologic changes in chicks after 16 to 30 egg passages.

From these findings it may be concluded that the degree of pathogenicity of the FMD virus in the chick played an important role

TABLE 4—The Pathogenic and Immunologic Response of Susceptible Cattle to the Egg-Adapted Foot-and-Mouth Disease Virus

Virus type	No. of chick and egg passages	Inoculum log mouse LD_{50}	Route of inoculation	Cattle No.	Reaction to virus from eggs	Serum protection indexes (days) 0	7	15	21	Reaction after challenge	Remarks
A	35 chick + 20 egg	7.3	i.d.-T	714	negative	0.6	>4.8	>5.7	N.T.	negative	immune
				715	negative	0.0	2.5	>4.7	N.T.	negative	immune
O	50 chick + 24 egg	6.1	i.d.-T	646	negative	0.0	>1.8	1.7	2.3	negative	immune
				653	T	0.5	>7.5	>7.8	>5.5	negative	immune
C	5 chick + 28 egg	5.9	s.c.	718	negative	0.0	<2.1	0.0	N.T.	T, 4 F	susceptible
			i.c.	719	negative	0.0	<2.1	3.7	N.T.	negative	immune
A				725						T, 3 F	susceptible
				728						T, 4 F	susceptible
					Controls for the challenge virus						
O				638						T, 4 F	susceptible
				685						T, 4 F	susceptible
C				717						T, 4 F	susceptible

T = tongue; F = feet; N.T. = not tested; i.d.-T = intradermal, tongue; s.c. = subcutaneous; i.c. = intracutaneous.

in its propagation in the embryonating eggs. Also, the use of the intravenous route of inoculation is probably successful because the virus is brought in contact with the heart tissue which has proved to be the particular site of virus localization in this host.

Despite the number of passages carried out with the 3 virus types in chicks, all types of the virus proved to lose their pathogenicity for cattle after 20 to 28 passages in eggs. Five of the 6 cattle tested were found to have satisfactory serum-protection indexes and proved to be immune to challenge with bovine virus.

The satisfactory concentration of virus in the embryonic tissues, besides its reasonable modification, may encourage use of the chicken embryo for production of a modified live-virus vaccine against FMD. However, much more information must be obtained from the experiments on cattle, and further investigations will have to be performed on several points, e.g., the duration of immunity, the interference between 2 or more modified viruses, the excretion of these viruses from inoculated animals, and the contact infection between vaccinated and nonvaccinated cattle.

SUMMARY

Two Brazilian strains of foot-and-mouth disease virus types A and C, and 1 Venezuelan strain of type O, which had been maintained in chicks for 5 to 50 passages, were successfully propagated in embryonating eggs for 20 to 30 passages.

The 3 egg-adapted virus types underwent a marked loss of pathogenicity for cattle. Four of 5 cattle that did not react to inoculation with these strains proved to have developed a strong immunity as judged by the antibody titer of their serums and by their resistance to challenge with bovine virus.

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SUMMARIO IN INTERLINGUA

Virus de Febre Aphthose. II. Propagation e Modification de Tres Typos Immunologic de Virus in Embryonate Ovos de Gallina

Duo racias brasilian de virus de febre aphthose del typos A e C e un racia venezuelan del typo O, le quales habeva essite mantenite in gallinettas a transverso inter 5 e 50 passages, esseva propagate a bon successo in embryonate ovos de gallina durante 20 a 30 passages.

In le adaptation a ovos, le tres typos de virus suffreva un perdita marcate de pathogenicitate pro bestial. Quatro ex cinque pecias de bestial que non reageva al inoculation de iste racias manifestava provas del disveloppamento de un forte immunitate a judicar per le titro de anticorpore in lor seros e per lor resistentia al provocation con virus bovin.

Foot-and-Mouth Disease Virus—Its Behavior in Cattle After Serial Passages in Chicken Embryos and Chicks

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THIS REPORT describes the reaction of cattle to foot-and-mouth disease virus (FMDV) after serial passages of 2 types in embryonating chicken eggs and in day-old chicks. Preliminary serial passages and infectivity tests had been reported by other investigators.

Gillespie[3] had previously propagated Holland type C of FMDV in embryonating chicken eggs after passages in chicks. He described the reactions in 6 cattle inoculated with the embryo-passaged virus. The cattle given embryo tissue from the 5th and 11th passages developed lesions of foot-and-mouth disease (FMD). The serums of animals given tissue from the 11th passage had neutralizing antibodies 17 days after exposure and were refractive to challenge. Two animals that were inoculated with material from the 25th passage did not develop signs of illness and were refractive. These observations prompted Gillespie to recognize the potential of the egg-propagated strain as a source of virus for vaccine and to emphasize the need for further investigation.

As a result of his studies, Komarov[8] concluded that adaptation of FMDV to chicken embryos had been achieved. Zahran,[14] following the work of Gillespie[4,5] and Skinner,[11] reported the successful propagation of FMDV in day-old chicks. All 3 types (A, O, and C) had been previously cultured by the Frenkel[2] technique. Of the bovine-passaged types only, type O was propagated by Zahran in the chick. Zahran,[14] working with the 25th transfer of chick-passaged type O FMDV, noticed that virus at this passage level caused only local lesions in a steer. The animal was refractory to challenge 19 days after initial exposure. Zahran[14] referred to this observation as an indication of possible adaptation, but he emphasized that no conclusion could be made until a larger number of animals were tested.

This report describes the reaction of cattle to FMDV following further serial passage of types C and O in embryonating chicken eggs and in day-old chicks. The 2 types, C and O, were adapted by Gillespie and Zahran, respectively.

MATERIALS AND METHODS

Type C FMDV at the 28th and 29th passages (described by Gillespie[3]) was selected for further passage in embryonating chicken eggs. Tissues from the 28th and 29th passages had been stored for 3 years in a dry-ice chest at −20 to −30 C. prior to use in these studies.

Type O FMDV of the 28th chick passage (described by Zahran[14]) was used for further passage in day-old chicks.

Pooled embryo or chicken heart and gizzard muscle were used to continue serial passage. Frenkel[2] medium was used as diluent in the preparation of tissue suspensions prepared by grinding the tissue with sand in sterile mortars followed by centrifugation for 15 minutes at 300 g.

Embryos were inoculated on the chorioallantoic membrane with 0.4 ml. of the supernatant fluid.

The complement-fixation (CF) test used to assay bovine serums was similar to that described by Marucci.[9]

Virus-neutralization (VN) tests were conducted according to the method described by van Bekkum.[12]

The ID_{50} of the challenge virus type C, Odoorn strain, was 2.4×10^9 per milliliter as determined in cattle and calculated by the method of Reed and Muench.[10] Titrations were made according to the method of Henderson.[7]

Guinea pigs used for infectivity tests were inoculated in the rear plantar pads.

Virus titrations were made in mice 9 to 10 days old, according to the method described by Skinner.[11] The plate method described by Brooksby and Wardle[1] was used with modifications.[6,13]

Five tenths milliliter of 10% tissue suspensions was used as inoculum for day-old Rhode Island x Leghorn chicks. The steers were imported from Ireland; the mice were mixed breeds of white and brown.

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Results

Type C FMDV in Chicken Embryos

Mortality.—On the 1st passage after storage, only 1 of 9 embryos given the 28th passage material and 2 of 15 embryos given the 29th passage material died within 96 hours after inoculation; however, the virus regained its prestorage virulence for chicken embryos after 2 passages. Tissues from Gillespie's [3] 28th passage were used to continue serial transfer. Of 180 embryos used in 11 passages, 153 (85%) died within 96 hours following inoculation.

Lesions.—Marked discoloration was seen throughout the entire skin area of infected embryos in passages 29 through 44. This change in the skin accentuated the contrast in color of the beak and the toes. No such changes were seen in the noninoculated embryos. The feathers of the dead and the moribund specimens were easily detached.

Virus Titer of Embryo Tissue.—The virus titer of embryo tissue (heart-gizzard) as measured by the plate titration technique was 10^7 at the 38th and 39th passages and $10^{6.4}$ and 10^5 ID_{50} in the 40th and 44th passages, respectively.

Infectivity Tests.—Infectivity tests were performed in mice and cattle with the following results.

Mice.—Tissues of embryo passages 28, 29, 31, 32, 35, and 38 were infective for mice. The mouse LD_{50} of the 40th passage was $10^{8.7}$. Virus in tissue suspensions of mice inoculated with material from the 40th embryo passage was confirmed by CF as type C FMDV.

Cattle.—Two steers were inoculated intradermolingually (IDL) at multiple sites with 0.75 ml. of 20% heart-gizzard suspension of the 38th embryo passage, which had been stored in an ice chest at −30 C. for 3 days. Both animals showed a marked rise in body temperatures within 5 days after inoculation. Foot lesions indicated that generalization occurred in 1 of the 2 steers. Lesions in the other animal were limited to the tongue.

Six steers were used to test the infectivity of embryo material from the 40th passage (Table 1). Each of 4 steers was inoculated intradermally in the caudal fold with 0.25 ml.; each of 2 steers was given 0.75 ml. of 20% embryo heart-gizzard suspension IDL. Body temperatures were recorded daily for 16 days postinoculation (DPI). The temperatures of both steers inoculated IDL rose to 104 F. on the 2nd and 3rd DPI and returned to normal on the 6th DPI. However, a 2nd rise of temperature in 1 of the 2 steers was recorded on the 13th and 14th DPI. The temperatures of 3 steers inoculated intradermally in the caudal fold did not exceed 102.2 F. within 6 days following the inoculation. The temperature of the 4th steer in this group reached 104 F. on the 6th day after inoculation and returned to normal on the 8th day.

Two lesions, approximately 1 cm. in diameter, occurred at sites of inoculation in 1 of the steers inoculated IDL, and a single lesion was seen in the 2nd animal. None of the 4 steers inoculated intradermally in the caudal fold developed lesions or other evidence of infection except for temperature elevation in one.

The serums of all 6 steers had significant VN titers at 29 days following injection (Table 1).

The immunity of all 6 steers was challenged with type C FMDV (Odoorn) strain. At least 10^5 ID_{50} doses were given IDL. No local lesions were noticed in any animals following challenge or at the time of slaughter 31 days postchallenge. Daily tempera-

TABLE 1—Foot-and-Mouth Disease Virus—Immune Response in Cattle to Type C After 40 Serial Passages in Chicken Embryos

Steer No.	Route	Complement-fixation test† Preinoculation Titer	Complement-fixation test† Postinoculation‡ Titer	Virus neutralization test§ Preinoculation Titer	Virus neutralization test§ Postinoculation Titer	Challenge at postinoculation
1	IDL*	25.0	50.0	0	5.4	Refractory
2	IDL	25.0	50.0	0.2	3.7	Refractory
3	ID**	25.0	50.0	0	3.5	Refractory
4	ID	25.0	100.0	0.2	5.1	Refractory
5	ID	25.0	75.0	0	5.5	Refractory
6	ID	0	125.0	0.2	5.4	Refractory

NOTE: All intradermal injections were made in the caudal fold. * IDL = intradermolingual; ** ID = intradermal; † the highest dilution of serum giving 50% fixation; ‡ all postinoculation tests and challenge were done at 29 days; § the logarithmic difference between: (1) ID_{50} of virus plus normal serum and, (2) virus plus test serum.

ture readings were made from all 6 animals for 31 days postinjection, and the highest temperature recorded during this holding period was 101.3 F. Two cattle inoculated IDL, as virus controls, developed primary and secondary lesions of FMD.

TYPE O FMDV IN DAY-OLD CHICKS

Type O FMDV, passaged 28 times in chicks by Zahran,[14] was used as starting material to initiate further serial passage in day-old chicks through passage 47.

Depression and inappetence were observed in some chicks at passage levels 29 through 35. However, no signs were seen among the chicks inoculated with tissues from passages 36 through 47.

Mortality.—Mortalities occurred at each passage level 29 through 35 but decreased thereafter. The decreases in mortality paralleled a decrease in the severity of lesions, particularly those of the gizzard. A few chicks of each passage were killed at 2 DPI because Gillespie [3] and Zahran [14] had indicated that the virus titer in chick tissue was usually highest on the 2nd and 3rd DPI. All survivors were examined for lesions on the 4th or 5th DPI.

Lesions.—Lesions described by Zahran [14] were of focal or diffuse type. During the earlier passages, lesions observed in this study continued to be primarily focal, but a change in the character of the lesions was noticed at the 34th passage level. This change was characterized at 2 to 3 DPI by yellow discoloration and the development of diffuse rather than focal lesions throughout the musculature of the gizzard.

Four- to tenfold differences in the concentration of virus were noticed in tissues of closely related passages.

Infectivity Tests.—Infectivity tests were performed in mice, guinea pigs, and cattle with the following results.

Mice.—Gizzard muscle from chicks at 9 passages, including the 30th and 47th, was infective for mice.

Guinea Pigs.—Tissues taken from the 33rd, 40th, 45th, and 47th chick passages were not infective for guinea pigs.

Cattle.—One steer, inoculated IDL with gizzard muscle from chicks at the 33rd passage level, developed primary and secondary lesions of FMD.

One of 2 steers similarly inoculated with tissue of the 47th chick passage level developed secondary lesions; only primary lesions were noticed in the other animal.

DISCUSSION

Type C FMDV passaged 38 times in chicken embryos caused a generalized infection in 1 of 2 steers inoculated IDL. This finding was in contrast to those of Gillespie [3] who reported that 2 animals inoculated with tissue suspension at the 25th passage level failed to develop lesions but were subsequently shown to be refractory to challenge. An equally abrupt change in the behavior of the virus was noticed in this study wherein 6 cattle inoculated with tissues from the 40th serial transfer failed to develop lesions and were later refractory to challenge.

The mortality rate in embryos, the character of lesions, and the infectivity for mice of tissues from both the 38th and 40th passage levels indicated that there probably were no marked quantitative differences in the virus of tissues at 2 different passage levels. The results at the 40th passage level may, on the other hand, reflect the persisting qualitative changes first observed by Gillespie [3] at the 25th passage.

The decrease in pathogenicity for chicks of type O chick-propagated FMDV after the 35th passage apparently was not accompanied by a similar change in pathogenicity for cattle, as the latter proved susceptible to infected chick tissue at the 33rd and 47th passages. This observation on the pathogenicity for cattle is in contrast to that of Zahran.[14] He reported that a susceptible ox inoculated with the 25th chick-passaged type O virus developed primary lesions and was subsequently immune to challenge with homologous FMDV. Traub and Schneider [12] described a guinea pig-adapted strain of FMDV which after chicken embryo passage acquired greater virulence for cattle but became less infective for guinea pigs.

SUMMARY

Sixteen serial passages of type C foot-and-mouth disease virus (FMDV) were completed in embryonating chicken eggs starting with the 28th passage.

Of 180 chicken embryos inoculated with FMDV in 11 passages, 153 (85%) died within 96 hours following inoculation.

Two steers inoculated intradermolingually with infective material from the 38th passage developed a generalized form of foot-and-mouth disease. Six steers inoculated with infective materials from the 40th passage did not develop generalized infection but were refractory to challenge. Of

the latter, 2 had been inoculated intradermolingually and 4 intradermally.

Twenty serial passages of type O FMDV were completed in day-old chicks by the intramuscular route, starting with the 28th passage. Infective material from the 33rd and 47th passages produced a generalized form of the disease in cattle.

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Summario in Interlingua

Virus de Febre Aphthose—Su Comportamento in Bestial post Passages Serial per Embryones de Gallina e Gallinettas

Dece-sex passages de virus de febre aphthose typo C esseva completate serialmente in embryonate ovos de gallina, comenciante con le 28ve passage.

Ex 180 embryones de gallina inoculate con le virus in 11 passages, 153 (i.e. 85%) moriva intra 96 horas post le inoculation.

Duo tauros inoculate per via intradermolingual con material infective ab le 38ve passage disveloppava un forma generalisate de febre aphthose. Sex tauros inoculate con materiales infective ab le 40me passage non disveloppava un infection generalisate sed se provava refractori contra un provocation. Duo de iste sex habeva essite inoculate per via intradermolingual e quatro per via intradermal.

Vinti passages serial de typo O de virus de febre aphthose esseva completate in gallinettas de un die de etate per via intramuscular, comenciante con le 28ve passage. Material infective ab le 33tie e le 47me passage produceva un forma generalisate del morbo in bestial.

Effects of Chlorpromazine, Reserpine, and Ascorbic Acid in Resisting Heat Stress in Rats

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ALTHOUGH various reports on the effects of chlorpromazine and reserpine on pituitary-adrenal function have been somewhat conflicting, it has become evident that, to a certain extent, these compounds can protect animals against exposure to stress.

Decourt *et al.*[5, 6] conducted experiments on guinea pigs at 25 C. to 45 C. and found that chlorpromazine reduced the body temperature even when the environmental temperature was 43 C. Although the experiments in this report, performed under the same conditions, do not fully confirm the results and conclusions by Decourt *et al.*, these data seem to indicate positive protective action of chlorpromazine against the fatal effects of high temperature.[12]

Several other authors found that chlorpromazine and reserpine, as well as desoxycorticosterone or adrenal extract, increased the survival time of adrenalectomized rats exposed to elevated environmental temperature.[5, 7-9] An intramuscular (i.m.) injection of 25 mg. of chlorpromazine is said to diminish the blood changes within 30 minutes in man exposed to 43 to 45 C. and 40 to 42% humidity.[6]

Chlorpromazine and reserpine also are reported to inhibit the adrenal ascorbic acid depletion induced by various stress conditions.[15, 22] Although there are several suggestions that the drugs act on the pre-pituitary phase, the precise mechanism of the activity of the drugs is still speculative.[1, 2, 14, 15, 23-25] Moreover, the possible participation of ascorbic acid in steroid biosynthesis and in the action of corticotropin (ACTH) has not been precisely determined; neither is the mechanism of action of ascorbic acid as a therapeutic agent understood.

Salomon[21] administered ascorbic-1-C^{14} acid to hypophysectomized rats and identified all C^{14} in the adrenal gland as ascorbic acid. After the rats were given corticotropin, C^{14} entered the adrenal venous blood. Salomon[18] believes that adrenal synthesis of ascorbic acid is either negligible or nonexistent. This observation is in accord with data obtained by some other authors. However, an explanation is still needed for the occurrence of high concentrations of ascorbic acid in the adrenal cortex of the normal animal. It has become evident that large doses of ascorbic acid not only prevent adrenal enlargement caused by stress but may also cause a reduction in the size of the gland.[25]

Weaver[26] claimed that oral administration of 100 mg. of ascorbic acid daily to workers exposed to excessive heat and humidity, sufficient to cause heat prostration, prevented the appearance of symptoms of illness due to hyperthermia. Some clinicians consider injections of ascorbic acid helpful and valuable in the treatment of some infectious diseases. This view seems to be supported by reports indicating that a bacterial polysaccharide complex evokes depletion of adrenal ascorbic acid.[13, 27, 28] It is worthwhile mentioning the work by Zagaievski,[29] who demonstrated that the susceptibility of ducklings to *Salmonella typhimurium* infection increased with heat and with hypervitaminosis C.

In the present investigations an attempt was made to compare the action of chlorpromazine, reserpine, and ascorbic acid on the pituitary-adrenal axis and to determine whether any of these drugs are effective in increasing the survival rate of normal rats exposed to high environmental temperature.

MATERIALS AND METHODS

Adult male and female rats of the Sprague-Dawley strain, weighing 200 to 350 Gm., were used in all experiments. All animals, maintained under the same conditions, were given Purina chow and tap water. For each experiment, rats of the same sex and similar age were used.

To produce heat stress, animals were restrained in individual wire tubes in a heat chamber at 43 C. and relative humidity of 54%. The body temperature was measured with thermocouples inserted rectally to a distance of 3 cm.* The duration of the hyperthermic stress period for any given experiment was arbitrarily chosen as the time necessary to produce death in 50% of the untreated rats.

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* The 12-channel thermistor telethermometer used in these experiments was built and calibrated by Mr. Arthur Stattelman.

TABLE 1—Effects of Daily Handling Upon Adrenal Ascorbic Acid, Adrenal Weight, and Body Weight in Rats at Room Temperature

Group	Treatment	No. rats	Adrenal ascorbic acid (mg./100 Gm. fresh tissue)	Adrenal weight (mg.)	Body weight (Gm.)
1	Controls, not handled (Saline solution, 1 cc., i.m.)	11	360.73	72.2	191.0
2	Handled for 40 days (Saline solution, 1 cc., i.m.)	11	429.76 P<0.005	64.0 P<0.005	190.0 P>0.5

P = compared with the control group; computed by Mrs. Onati by the analysis of variance.

In the main experiment, all rats were allotted to 6 groups: (1) control, given saline solution; (2) chlorpromazine, 5 mg./kg.; (3) chlorpromazine, 20 mg./kg.; (4) reserpine, 10 μg./kg.; (5) reserpine, 20 μg./kg.; and (6) ascorbic acid, 100 mg./kg. All compounds were administered intramuscularly 1 hour prior to the onset of stress. Ascorbic acid was injected at 24 hours and again at 1 hour before exposure to heat stress.

At the time when 50% of control animals were dead (time of median control death), each surviving rat was killed by a blow on the head. Both adrenal glands were immediately removed, weighed together, and analyzed for adrenal ascorbic acid concentration. Analyses of ascorbic acid were performed spectrophotometrically by the modified method of Roe and Keuther.[6, 16, 17]

PROCEDURE AND RESULTS

This investigation did not confirm the conclusion of Holzbauer and Vogt,[11] who claim that chlorpromazine causes depletion of the ascorbic acid in the adrenal cortex. In preliminary experiments carried on with a limited number of normal rats, chlorpromazine (25 mg./kg.) and reserpine (50 μg./kg.) produced no greater effect on the concentration of adrenal ascorbic acid than did saline solution. Data published by other authors support this observation.[18, 22]

It appears that the discrepancy between reported data obtained from experiments on stress may result from different psychologic conditions of the experimental animals which influence the degree of stress affecting the animal. This view is supported by the following observation and experiment.

At the beginning of this investigation, it was noticed that rats unaccustomed to the presence of or handling by man were somewhat more sensitive to high temperature than rats that had been handled and showed no fear of man. Initial analyses for the ascorbic acid content of the adrenal cortices of rats unaccustomed to man varied consistently and considerably from those of rats exposed to constant observation by man.

In order to test this observation, 2 groups of 1-month-old female rats were used for the experiment.* The animals were maintained in individual cages. Rats in 1 group (controls) were not touched; whereas, each rat from the other group was handled for 2.5 minutes every day. After 40 days, all rats of both groups were weighed and injected with 1 cc. of saline solution to simulate the process of injecting an experi-

* This experiment was carried out by Mrs. Luz Onati under the supervision of Dr. L. Meyer Jones and the author in the Department of Veterinary Physiology and Pharmacology, Iowa State University, Ames.

TABLE 2—Effects of Chlorpromazine, Reserpine, and Ascorbic Acid on Rectal Temperature in Surviving Rats at 43 C. and 54% Humidity

Group	Treatment	No. rats	Time in minutes 0	30	45	60	75*
1	Control (saline solution)	48	37.39 ± 0.14**	40.47 ± 0.14	41.65 ± 0.14	42.45 ± 0.14	42.87 ± 0.15
2	Chlorpromazine (5 mg./kg., i.m.)	16	35.92 ± 0.13 P_1<0.001†	38.96 ± 0.27 P_1<0.001	40.27 ± 0.32 P_1<0.001	41.31 ± 0.25 P_1<0.001	41.95 ± 0.11 P_1<0.001
3	Chlorpromazine (20 mg./kg., i.m.)	8	35.42 ± 0.26 P_2>0.1‡	38.25 ± 0.39 P_2>0.1	39.74 ± 0.31 P_2>0.1	41.00 ± 0.39 P_2>0.1	41.78 ± 0.21 P_2>0.1
4	Reserpine (10 μg./kg., i.m.)	16	36.83 ± 0.15 P_1<0.01	40.04 ± 0.15 P_1<0.05	41.27 ± 0.13 P_1>0.05	42.11 ± 0.16 P_1>0.1	42.55 ± 0.14 P_1>0.1
5	Reserpine (20 μg./kg., i.m.)	8	36.93 ± 0.19 P_1>0.05	39.45 ± 0.17 P_1<0.005	40.87 ± 0.13 P_1<0.005	41.89 ± 0.20 P_1<0.95	42.35 ± 0.15 P_1<0.05
6	Ascorbic acid (100 mg./kg., i.m.)	12	37.11 ± 0.15 P_1>0.1	39.99 ± 0.19 P_1<0.05	41.19 ± 0.13 P_1<0.05	41.92 ± 0.16 P_1<0.05	42.33 ± 0.12 P_1<0.05

* LD_{80} exposure time for control rats. ** Mean temperature ± standard deviation. † Compared with the control group No. 1. ‡ Compared. with the group No. 2.

TABLE 3—Effects of Chlorpromazine, Reserpine, and Ascorbic Acid on Adrenal Ascorbic Acid Level, Adrenal Weight, and Survival of Rats at 43 C. and 54% Humidity

Group	Treatment	No. rats		Adrenal ascorbic acid		Adrenal weight surviving		
		Used	Surviving	mg./100 Gm. fresh tissue	s.d.	mg.	s.d.	%
0	Control (normal subjects at 26 C.)	10	....	423.00	± 4.79	49.20 ± 0.79		.....
1	Control (saline solution, i.m.; at 43 C.)	48	24	180.73	± 6.70	64.45 ± 2.11		50.00
				$P_0<0.001$		$P_0<0.001$		.....
2	Chlorpromazine (5 mg./kg., i.m.)	16	13	257.30	± 6.45	52.69 ± 1.33		81.25
				$P_1<0.001$		$P_1<0.001$		$P_1<0.05$
3	Chlorpromazine (20 mg./kg., i.m.)	8	7	273.00	± 6.99	49.71 ± 1.02		87.50
				$P_2>0.1$		$P_2>0.01$		$P_1<0.05$
4	Reserpine (10 μg./kg., i.m.)	16	11	230.54	± 8.22	54.91 ± 1.42		68.75
				$P_1<0.001$		$P_1<0.001$		$P_1>0.05$
5	Reserpine (20 μg./kg., i.m.)	8	6	265.67	± 6.73	49.67 ± 0.98		75.00
				$P_4<0.01$		$P_4<0.05$		$P_1>0.05$
6	Ascorbic acid (100 mg./kg., i.m.)	12	9	254.33	± 8.08	58.00 ± 2.19		75.00
				$P_1<0.001$		$P_1<0.05$		$P_1>0.05$

The small figure beside P indicates the number of the other group with which this is compared.

mental drug. One hour after injection, all rats were killed by a blow on the head and determinations of adrenal ascorbic acid and adrenal weight were made as specified previously.

As indicated (Table 1), an ordinary but (to the animal) unaccustomed experimental procedure, such as an i.m. injection or weighing, may cause effects of stress as judged by the significant depletion of adrenal weight in untrained, frightened animals. When exposed to the same conditions, rats accustomed to handling may show no detectable stress effects at all.

In the experiments at the higher ambient temperature (43 C.), all 3 drugs provided some protection to rats in withstanding the stress of hyperthermia (Fig. 1). Fifty per cent of the control rats placed in individual restraining wire tubes at 43 C. and a relative humidity of 54% died within 75 minutes of exposure. During the same exposure time to the same conditions, the survival rate of treated rats in all experimental groups was higher than in the control group. However, the protection obtained with reserpine and ascorbic acid were statistically questionable when analyzed by the *Chi*-square method. The higher doses of chlorpromazine or reserpine produced insignificant increases in the survival rate at the lower dosage level.

Among the surviving rats, those treated with chlorpromazine had the lowest body temperatures; rats treated with the largest dose of reserpine and with ascorbic acid had lower body temperatures than the untreated rats (Table 2). The body temperatures of all dying rats were similar at the moment of death. The statistical analyses of the data (Table 2) presented difficulties. The problem arose because the drugs had to be given an hour before the experiment in order to be effective during the observation period and, under the conditions of the experiment, body temperature could not be recorded until the heat stress began. Thus, Table 2 shows that the initial temperatures of the rats treated with chlorpromazine were reduced 1.5 to 2.0 degrees below that of the controls. No method of statistical treatment of these data would be entirely satisfactory.

None of the drugs used prevented a decrease in the adrenal ascorbic acid level, but the rate of depletion was significantly less in rats of each of the treated groups than in the controls (Fig. 2). The opposite was true for adrenal weight; *i.e.*, the compensatory increase in adrenal weight re-

TABLE 4—Effects of Chlorpromazine on Rectal Temperature in Rats at Room Temperature (26 C.) and 54% Humidity

Group	Treatment	No. rats	Time of restraining (minutes)			
			0*	30	60	90
7	Control (saline solution, i.m.)	6	37.60 ± 0.14**	37.58 ± 0.26	37.47 ± 0.23	37.32 ± 0.21
8	Chlorpromazine (20 mg./kg., i.m.)	6	35.62 ± 0.17 P<0.005†	34.92 ± 0.26 P<0.005	34.70 ± 0.22 P<0.005	34.43 ± 0.20 P<0.005

* 1 hour after injection. ** Mean temperature ± standard deviation. † P = Compared with control group 7.

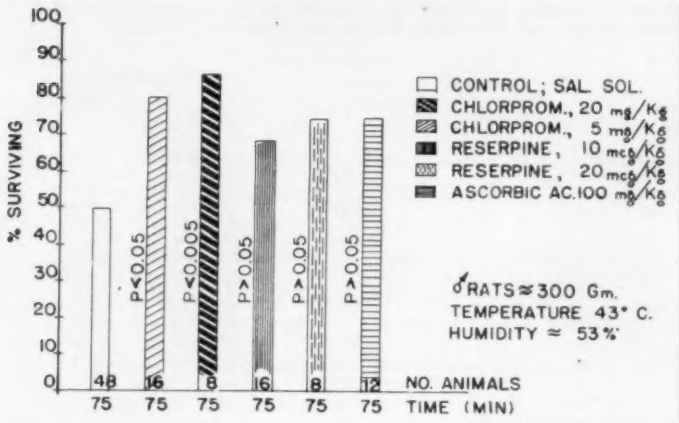

Fig. 1—Effects of intramuscular injections of chlorpromazine, reserpine, and ascorbic acid on survival at high temperature.

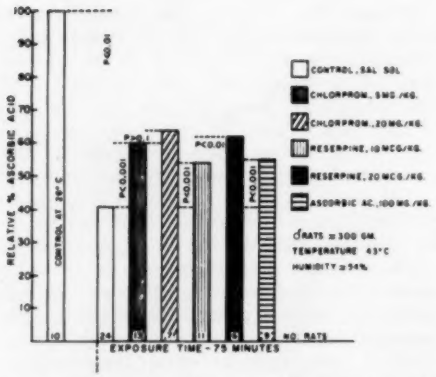

Fig. 2—Effects of chlorpromazine, reserpine, and ascorbic acid on adrenal ascorbic acid levels.

sulting from hyperthermic stress was less in the treated than in the control rats (Table 3).

After this experiment, the question was raised whether or not the restraining of rats could stimulate an animal via the pituitary-cortical system, *i.e.*, whether restraining of rats in individual, tight, wire tubes could be considered a predisposing factor for stress of hyperthermia.

The experiment was carried on in the same conditions as described above, but at room temperature (26 C.). Two groups of rats were used: controls, given i.m. saline solution, and principals, given i.m. chlorpromazine. These animals were put in restraining tubes for 90 minutes. Under these conditions, the average rectal temperature of the rats given chlorpromazine (20 mg./kg.) was significantly lower than that of the controls. In both groups of animals, a slight progressive decrease of temperature was noticed (Table 4). At the end of the experiment, all rats were killed for individual determination of adrenal ascorbic acid and adrenal weight. As would be expected, restraining of the animals at room temperature caused a decrease in the concentration of adrenal ascorbic acid and a compensatory increase in adrenal weight (Fig. 4). However, the response of restrained rats tranquilized with chlorpro-

TABLE 5—Effects of Chlorpromazine on Adrenal Ascorbic Acid and Adrenal Weight in Rats Restrained at Room Temperature (26 C.) and 54% Humidity

Group	Treatment	No. rats	Adrenal ascorbic acid mg./100 Gm. fresh tissue s.d.	Adrenal weight mg. s.d.
0	Control (normal subjects, not restrained)	10	423 00 ± 4.79 $P_7<0.005$*	49.20 ± 0.79 $P_7<0.005$
7	Control (saline solution, i.m.)	6	252.17 ± 13.28 $P_8<0.005$	67.66 ± 3.58 $P_8<0.005$
8	Chlorpromazine (20 mg./kg., i.m.)	6	326.33 ± 11.57 $P_0<0.005$	54.17 ± 2.01 $P_0<0.05$

* The small figure beside P indicates the number of the other group with which this is compared.

mazine was comparatively less than that of the restrained untreated controls, but statistically significant (Table 5).

DISCUSSION

It is known that if animals which have been reared under uniform conditions are suddenly moved to an unfamiliar environment, they usually are frightened, eat and drink less, and lose body weight. Steinberg and Watson [24] reported that chlorpromazine can inhibit the reaction of rats to unfamiliar surroundings. There are some suggestions also that restrained rats have consistently higher metabolic rates than free rats. Frankel [7] has found that mean time to death at high temperatures was significantly longer for free rats than for restrained ones.

In considering these data, the assumption has been made that changes in environmental conditions or restraining of animals can be stressful for animals in the sense of Selye. This so-called "psychological stress" may serve as a predisposing factor when the other kind of stress (hypothermia) comes into action. Probably it causes stimulation via the central nervous system and the pituitary-cortical axis.

Knowing that ascorbic acid assays have shown great variation in the hands of competent scientists, great care was taken in this investigation to obtain consistent and reproducible results. The 1st experiment confirmed the suspicion that the psychological stress factor must be considered in the management of experimental animals used for measuring the adrenal ascorbic acid levels. Rough, unnecessary handling of rats unaccustomed to these procedures, or to man at all, acts as a stress factor that promptly drains the ascorbic acid from the adrenal cortex. Variations in animal management would appear to be at least a partial explanation for previous disparities in data on adrenal ascorbic acid determinations. Throughout the experiment, great care was taken to avoid the psychologic stress phenomenon.

All 3 drugs used were found to be of some benefit to rats in resisting heat stress, even though chlorpromazine, reserpine, and especially ascorbic acid act by different mechanisms, according to some investigators.[10] Chlorpromazine proved to be more effective than reserpine and ascorbic acid in halting both depletion of adrenal ascorbic acid and

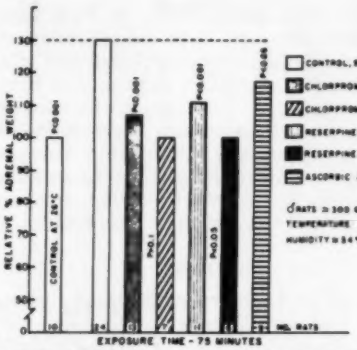

Fig. 3—Effects of chlorpromazine, reserpine, and ascorbic acid on adrenal gland weight.

increasing of adrenal weight in rats exposed to 43 C. However, it should be pointed out that neither drug fully prevented occurrence of these phenomena in response to severe heat stress. This observation is not completely in accord with data obtained by Mahfouz *et al.*,[15] who found reserpine much more effective than chlorpromazine. Under the experimental conditions of the present studies, chlorpromazine conferred the highest survival rate and the best protection to rats against the fatal effects of heat.

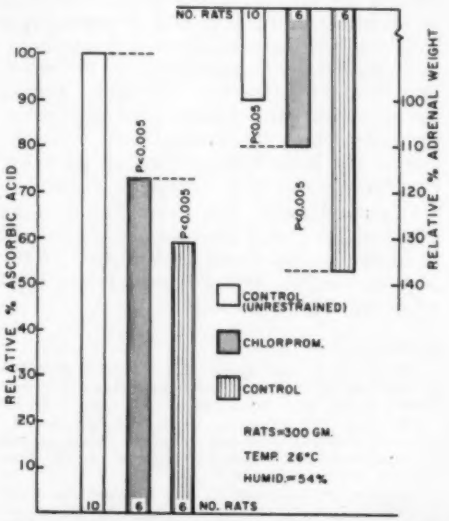

Fig. 4—Effects of chlorpromazine (20 mg./kg., i.m.) on adrenal ascorbic acid and adrenal gland weight in rats restrained at room temperature.

In this heat stress study, as with some other kinds of stress, there exists a relationship between the adrenal ascorbic acid concentration and adrenal weight on one hand and the degree of resistance to heat stress on the other. Presumably, there is an inversely proportional relationship between the degree of pituitary-cortical stimulation and the rate of survival following exposure to the severe stress of high temperature.

SUMMARY

A small preliminary experiment demonstrated that rats accustomed to daily handling did not experience psychologic stress similar to that which reduced the adrenal cortical level of ascorbic acid in the control rats unaccustomed to handling by man.

In the principal investigation, an attempt was made to compare the action of chlorpromazine, reserpine, and ascorbic acid on the adrenal ascorbic acid, adrenal weight, and survival rate in normal rats exposed to heat (43 C.). None of the drug fully prevented a decrease in the adrenal ascorbic acid level and a compensatory increase of adrenal weight. However, all 3 drugs provided some protection in withstanding the stress of high temperature. Fifty per cent of the rats placed in individual restraining tubes at 43 C. and a relative humidity of 54%, died within 75 minutes of exposure. During the same exposure time to the same conditions, the survival rate of treated rats was 87% for the rats injected intramuscularly with chlorpromazine, 75% for reserpine, and 75% for ascorbic acid. Among the surviving rats, those treated with chlorpromazine had the lowest body temperatures. The body temperatures of all dying rats was similar at the moment of death.

Chlorpromazine has been found more effective than reserpine and ascorbic acid in protecting rats against depletion of the adrenal ascorbic acid and against the fatal effects of high temperature.

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SUMMARIO IN INTERLINGUA

Le Effectos de Chlorpromazina, Reserpina, e Acido Ascorbic Super le Resistentia a Stress Thermal

Un parve experimento preliminari demonstrava que rattos accostumate a manipulationes quotidian non experientiava un stress psychologic simile a illo que reduceva le nivello adrenocortical de acido ascorbic in le rattos de controlo non-accostumate al manipulation per homines.

In le studio principal, un essayo esseva facite de comparar le action de chlorpromazina, reserpina, e acido ascorbic super le acido ascorbic adrenal, le peso adrenal, e le superviventia in rattos normal exponite a calor (43 C). Nulle del 3 drogas totalmente preveniva un reduction in le nivello de acido ascorbic adrenal e del augmentation compensatori del peso adrenal. Nonobstante, cata un del tres drogas provideva un certe protection contra le stress de alte temperatura. Cinquanta pro cento del rattos ponite in tubos individual de restringimento a 43 C con un humiditate relative de 54% moriva intra 75 minutas de exposition. Durante le mesme tempore de exposition al mesme conditiones le superviventia del rattos tractate esseva 87% in le caso del rattos recipiente chlorpromazina per via intramuscular, 75% in le caso del rattos recipiente reserpina, e 75% in le caso del rattos recipiente acido ascorbic. Inter le rattos supervivente, illos tractate con chlorpromazina habeva le temperaturas corporee le plus basse. Le temperaturas corporee de omne le rattos que moriva, esseva simile al tempore de lor morte.

Ha essite trovate que es plus efficace que reserpina e acido ascorbic in proteger rattos contra le depletion del acido ascorbic adrenal e contra le effectos letal del alte temperatura.

Effects of Chlorpromazine and Ascorbic Acid in Rats During Simulated Transportation Conditions at Normal and High Temperatures

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SOME tranquilizing drugs and ascorbic acid have been found to decrease effects of stress induced by various means. A short review of literature and the effects of chlorpromazine, reserpine, and ascorbic acid in resisting heat stress in rats have been previously summarized.[4]

From field trials, claims have been made that certain tranquilizing drugs diminish stress effects in transported animals.[1,3] In the present investigation, an attempt has been made to find experimental confirmation of this idea, using chlorpromazine as the oldest and most typical tranquilizing compound.

MATERIALS AND METHODS

All tests were performed on healthy, normal rats of Sprague-Dawley strain, weighing about 250 Gm. The animals were maintained under the same conditions and fed Purina chow and tap water ad libitum. Male rats of the same age were chosen for each experiment except the last experiment, in which both males and females were used. In the latter case, data were analyzed separately for each sex.

In order to simulate agitation in a moving truck, control and experimental rats were shaken in a small elliptical pattern at the rate of 165 excursions per minute. The pattern was 1.5 cm. in the vertical direction and 2.8 cm. in the horizontal direction. After 2, 4, 6, and 12 hours of shaking, rats were killed by a blow on the head for determinations of adrenal weight, adrenal ascorbic acid, and loss of weight.

Chlorpromazine was administered intramuscularly 1 hour prior to the onset of shaking. Ascorbic acid was injected intramuscularly twice, 24 hours and 1 hour before shaking.

Analyses of ascorbic acid content in the adrenals were performed as previously described.[4]

PROCEDURE AND RESULTS

At the beginning of the 1st experiment, 16 rats (group 1) were used to obtain normal values of adrenal ascorbic acid and adrenal weight for the entire group of animals.

Significant depletion of adrenal ascorbic acid was found after 2 hours of shaking (Fig. 1). However, the concentration of adrenal ascorbic acid was significantly higher within the first 4 hours of shaking in the rats treated with chlorpromazine. At

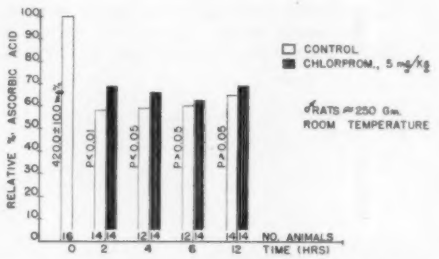

Fig. 1—Effect of chlorpromazine (i.m.) on adrenal ascorbic acid during shaking.

the end of the 4th hour and thereafter, the differences were not statistically significant. In both groups, after the initial stress of shaking, the adrenal ascorbic acid levels increased slowly toward the physiologic level.

As would be expected, adrenal weights in the rats of both groups responded conversely to the concentration change of the adrenal ascorbic acid and increased during the experiment. The tranquilized rats had a smaller increase, but the differences between the treated and untreated rats were not statistically significant (Fig. 2).

An increasing loss of body weight pro-

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portional to the duration of shaking was found in both groups of rats, but the weight loss was much less in the rats injected with chlorpromazine and was statistically significant (Fig. 3).

The data of the 1st experiment are shown (Table 1).

In the 2nd experiment, the controls (group 5) and principals (group 6) were shaken for 4 hours, which was the length of time producing the most pronounced stress effects observed during experiment I. The principals were premedicated with ascorbic acid. Physiologic values for animals used in this experiment were determined on 8 normal rats (group 4).

Surprisingly, there was no difference in the mean loss of body weight in control and treated rats; significant differences occurred in adrenal ascorbic acid and in adrenal weights (Fig. 4). These 2 ratios (control: treated) were inversely proportional, as commonly occurs under the conditions of stress (Table 2).

In the 3rd experiment, control rats and rats pretreated with chlorpromazine and ascorbic acid were shaken in a heat chamber at 40 C. and 54% humidity until 50% of the controls were dead (4 hours and 20 minutes). At this time, 81% of the treated animals were alive (Fig. 5).

In this experiment, male and female rats were used to identify any sex difference.

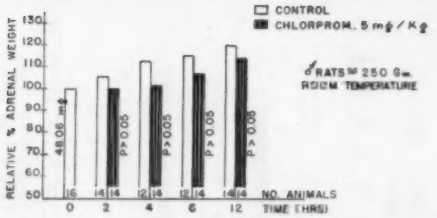

Fig. 2—Effect of chlorpromazine (i.m.) on adrenal weight during shaking.

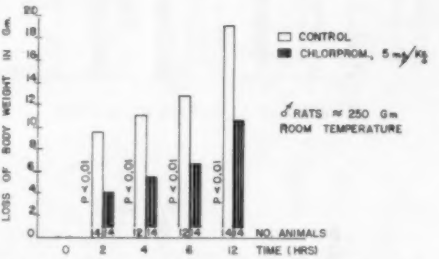

Fig. 3—Effects of chlorpromazine (i.m.) on body weight loss during shaking.

The data on the concentrations of adrenal ascorbic acid and adrenal weights between the 2 sexes are presented separately (Table 3). In spite of the sex differences, adrenal ascorbic acid of treated rats exceeded that of the controls; the opposite was true for adrenal weight. The body weight loss of treated rats was also much lower (Fig. 6).

TABLE 1—Effects of Chlorpromazine on Adrenal Ascorbic Acid, Adrenal Weight, and Body Weight During Shaking at Room Temperature (26 C.) and 54% Humidity

Group	Shaking time (hours)	Treatment	No. rats	Adrenal ascorbic acid mg./100 Gm. fresh tissue s.d.	Adrenal weight mg. s.d.	Loss of body weight Gm. s.d.
1	0	Control (without shaking)	16	420.50 ± 10.19	48.06 ± 1.66	.....
2	2	Control (saline solution, i.m.)	14	247.93 ± 7.72 P<0.01	50.71 ± 2.98 P>0.05	9.50 ± 0.93 P<0.01
3	2	Chlorpromazine (5 mg./kg., i.m.)	14	290.07 ± 13.01	47.86 ± 2.75	4.07 ± 0.67
2	4	Control (saline solution, i.m.)	12	248.83 ± 10.54 P = 0.02	54.08 ± 2.93 P>0.05	11.00 ± 1.21 P<0.01
3	4	Chlorpromazine (5 mg./kg., i.m.)	14	279.14 ± 7.97	49.00 ± 2.35	5.28 ± 0.62
2	6	Control (saline solution, i.m.)	12	254.50 ± 9.09 P>0.05	55.08 ± 1.99 P>0.05	12.75 ± 1.13 P<0.01
3	6	Chlorpromazine (5 mg./kg., i.m.)	14	264.21 ± 8.67	51.36 ± 2.19	6.64 ± 0.92
2	12	Control (saline solution, i.m.)	14	275.78 ± 9.89 P>0.05	57.71 ± 2.19 P>0.05	18.93 ± 1.15 P<0.01
3	12	Chlorpromazine (5 mg./kg., i.m.)	14	292.64 ± 9.78	55.07 ± 1.99	10.64 ± 0.70
		Total No. rats used	124			

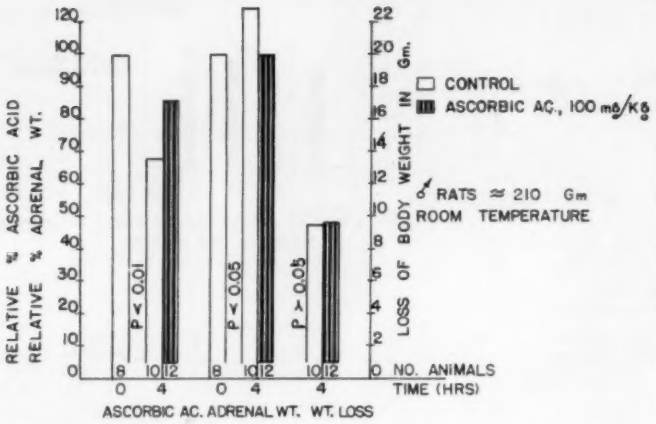

Fig. 4—Effects of ascorbic acid (i.m.) on adrenal ascorbic acid, adrenal weight, and body weight during shaking.

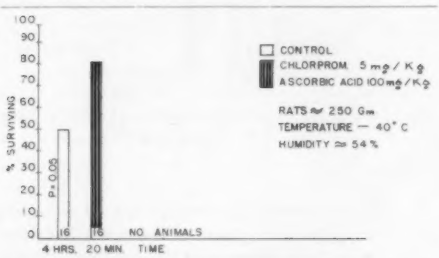

Fig. 5—Effects of chlorpromazine and ascorbic acid on survival during shaking at high temperatures.

Discussion

In the present experiments, it was found that the shaking of rats in a machine to simulate transportation in a moving truck or railroad car can be stressful enough to cause stimulation of the pituitary-adrenal axis, as indicated by adrenal ascorbic acid depletion and increased adrenal weight. Under the conditions of actual transportation, the effects of a stressful situation probably may be enhanced by additional environmental factors, such as crowding and tiredness, that have long been recognized as predisposing factors to the "shipping" diseases. Undoubtedly, one such factor is an increased ambient temperature, as employed in these experiments.

Chlorpromazine has been reported to suppress the depletion of adrenal ascorbic acid induced by various stressful stimuli.[2,4-6] A somewhat similar effect also has been recorded after the administration of ascorbic acid to rats.[4,7] These anti-stress effects of chlorpromazine and ascorbic acid were evident in rats which were shaken at room temperature and also at 43 C.

It is of interest that rats given chlorpromazine alone had much less body weight loss than the untreated rats. This observation is in accord with data obtained by Foley *et al.*[3] for shipping cattle. Unexpectedly, ascorbic acid was without effect on body weight changes of rats as compared with that of the untreated rats. These data, however, are only of relative value, because no typical metabolic determinations were made.

The results of the present investigations suggest that, for rats under simulated transport conditions, chlorpromazine possesses some protective action against excessive

TABLE 2—Effects of Ascorbic Acid on Adrenal Ascorbic Acid, Adrenal Weight, and Body Weight after Shaking at Room Temperature (26 C.) and 54% Humidity

Group	Treatment	No. rats	Adrenal ascorbic acid mg./100 Gm. fresh tissue s.d.	Adrenal weight mg. s.d.	Loss of body weight Gm. s.d.
4	Control (without shaking)	8	427.12 ± 12.94	48.75 ± 3.61	.....
5*	Control (saline solution, i.m.)	10	253.80 ± 11.10 P<0.01	55.60 ± 3.63 P<0.05	9.40 ± 1.09 P>0.05
6*	Ascorbic acid (100 mg./kg., i.m.)	12	370.67 ± 6.93	48.00 ± 2.18	9.83 ± 0.72
	Total No. rats used	30			

* All rats in groups 5 and 6 were shaken for 4 hours.

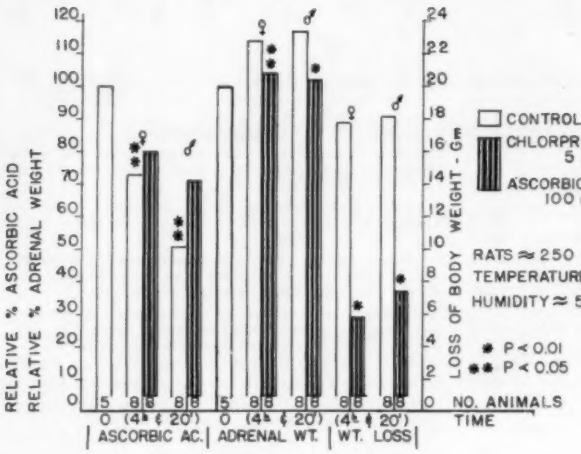

Fig. 6—Effects of chlorpromazine and ascorbic acid (i.m.) on adrenal ascorbic acid, adrenal weight, and body weight during shaking at high temperature.

weight losses, excessive depletion of the adrenal ascorbic acid, and the excessive mortality experienced at high temperature. The simultaneous administration of ascorbic acid with chlorpromazine seems to be beneficial because it augments the adrenal ascorbic acid level, particularly when the stress of agitation is enlarged by highly ambient temperature.

Summary

An attempt has been made to find experimental confirmation of the beneficial effects claimed clinically for the use of chlorpromazine and ascorbic acid in allaying the stress of transportation.

Normal rats were shaken in a small elliptical pattern, simulating the agitation in a moving truck or railroad car, for 2, 4, 6, and 12 hours. Rats given chlorpromazine intramuscularly (5 mg./kg.) and shaken at room temperature had significantly less body weight loss ($P<0.01$) than the control rats that were given physiologic saline solution intramuscularly. Furthermore, the concentration of adrenal ascorbic acid, as an index of stress, was significantly greater in the chlorpromazine-treated rats during the first 4 hours of shaking. Thereafter, the difference was not statistically significant.

In a similar experiment, rats were given ascorbic acid intramuscularly (100 mg./kg.). The treated rats had as much body weight loss as the controls, but their adrenal ascorbic acid levels were significantly higher ($P<0.01$).

In another experiment, rats pretreated with combined chlorpromazine-ascorbic acid and control rats given saline solution were shaken in a heat chamber (40 C. and 54% humidity) until 50% of the controls were dead. At this moment, 81% of the treated animals were alive. The adrenal ascorbic acid of the treated rats exceeded that of the control rats ($P<0.025$). The body weight loss of the treated rats was also significantly

TABLE 3—Effects of Chlorpromazine and Ascorbic Acid on Adrenal Ascorbic Acid, Adrenal Weight, and Body Weight After Shaking at 40 C. and 54% Humidity

Group	Treatment	No. rats	Sex	Adrenal ascorbic acid mg./100 Gm. fresh tissue	s.d.	Adrenal weight mg.	s.d.	Loss of body weight Gm.	s.d.
7	Control (at 26 C.) without shaking	5	♂	384.80	± 4.89	44.40	± 1.20	.....	
		5	♀	313.60	± 2.64	53.80	± 1.07	.....	
8*	Control (saline solution, i.m.; at 40 C.)	8	♂	196.87	± 3.91 P<0.025	52.37	± 1.50 P<0.01	18.25	± 1.14 P<0.01
		8	♀	231.87	± 8.03 P = 0.05	61.50	± 1.28 P<0.05	17.87	± 0.93 P<0.01
9*	Chlorpromazine (5 mg./kg., i.m.) and Ascorbic acid (100 mg./kg., i.m.)	8	♂	274.75	± 7.47	45.62	± 1.05	7.50	± 0.76
		8	♀	252.50	± 5.46	56.25	± 1.47	55.87	± 0.88
	Total No. rats used	40							

P = compared with the group 3 within the same sex.
* All rats in groups 8 and 9 were shaken for 4 hours and 20 minutes.

less ($P<0.01$) than that of the control animals.

These experiments showed that, under simulated transport conditions, chlorpromazine protects rats against excessive weight losses, excessive depletion of the adrenal ascorbic acid, and the excessive mortality experienced at high temperature. The simultaneous administration of ascorbic acid with chlorpromazine seemed to promote anti-stress effects, particularly at high temperatures.

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SUMMARIO IN INTERLINGUA

Effectos de Chlorpromazina e de Acido Ascorbic in Rattos sub Simulate Conditiones de Transporto a Normal e Elevate Temperaturas Ambiental

Esseva interprendite le tentativa de obtener un confirmation experimental del these, formulate a base de observationes clinic, de effectos benefic resultante del uso de chlorpromazina e acido ascorbic in le alleviation del stress de transporto.

Rattos normal esseva succussate in un miere orbita elliptic, simulante le agitation in un camion in motion o in un wagon de ferrovia. Le procedura esseva continuate durante 2, 4, 6, e 12 horas. Rattos tractate con chlorpromazina per via intramuscular (5 mg per kg de peso corporee) e succussate al temperatura normal de interior manifestava significativemente plus basse perditas de peso corporee (P minus que 0,01) que le rattos de controlo que habeva essite tractate con un physiologic solution salin per via intramuscular. In plus, le concentration de acido ascorbic adrenal (prendite como indice de stress) esseva significativemente plus alte in le rattos tractate con chlorpromazina durante le prime 4 horas del succussation. Postea le differentia cessava esser statisticamente significative.

In un simile experimento, rattos esseva tractate con acido ascorbic per via intramuscular (100 mg per kg de peso corporee). Iste rattos habeva le mesme perdita de peso corporee como le rattos de controlo, sed lor nivellos de acido ascorbic adrenal esseva significativemente plus alte (P minus que 0,01).

In un altere experimento, rattos pretractate con un combination de chlorpromazina e acido ascorbic e rattos de controlo tractate con solution salin esseva succussate in un camera de calor (40 C, con un humiditate de 54%) usque 50% del animales de controlo esseva morte. A iste momento, 81% del tractate animales esseva vive. Le nivello adrenal de acido ascorbic del tractate rattos excedeva illo del rattos de controlo (P minus que 0,025). Le perdita de peso corporee del tractate rattos esseva etiam significativemente minus marcate (P minus que 0,01) que illo del animales de controlo.

Iste experimentos monstrava que—sub simulate conditiones de transporto—chlorpromazina protege rattos contra excessive perdita de peso, contra le excessive depletion del acido ascorbic adrenal, e contra le excessive mortalitate que occurre a alte temperaturas. Le simultanee administration de acido ascorbic e chlorpromazina pareva promover le effecto anti-stress, particularmente a alte temperaturas.

Thyroid-Active Agents and Heat Stress in the Rat

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RECENT REPORTS indicate that chlorpromazine and reserpine possess some antithyroidal properties. Chlorpromazine was shown to decrease the radioiodine uptake of the thyroid and to produce goiter with depletion of the intrathyroidal store of iodine in mature female rats.[13] Pretreatment of rats or ground squirrels with 3-5-3'-triiodothyropropionic acid, the synthetic thyroxine analog, inhibits the chlorpromazine-induced hypothermia.[12]

The I^{131} uptake in the thyroid was decreased by the administration of reserpine. The antithyroid action of reserpine is suggested to be a result of its effect upon the elaboration or release of thyrotropin by the pituitary.[8,15] Probably, the central nervous system can influence this action, and the effects of it may be transmitted to the thyroid through the pituitary or adrenal glands.[1,19]

Some authors suggested also that the depressed iodine uptake, in part at least, was due to a direct action of adrenal cortical hormones upon the thyroid.[2,6,16]

During the hypothyroidism of myxedema and after surgical thyroidectomy or thiouracil treatment, the production of adrenocortical steroids appears to be reduced with the simultaneous involution of the adrenal glands. The opposite effect seems to be true in hyperthyroidism.[5,10,11] The mechanism of the functional relationship between the pituitary-adrenal axis and thyroid is not clearly understood, and it is probable that a clear definition of these or other factors is necessary.[4,6]

It is interesting to cite data indicating that several stressing agents, such as epinephrine, formalin, or typhoid vaccine, cause a decrease in the radioiodine uptake and promote the excretion of iodine in urine.[9]

The depletion of adrenal ascorbic acid in stress has been demonstrated by several authors, but there are some indications that the thyroid also can influence adrenal ascorbic acid level. Thyroid administration in guinea pigs raises their requirements of ascorbic acid. Rabbits and rats with hyperthyroidism had increased excretion of ascorbic acid in their urine.[3,7] Administration of ascorbic acid was said to protect guinea pigs against thyroid hypertrophy when the pituitary thyrotropic factor was injected.[20] However, surgical thyroidectomy or administration of thiouracil causes involution of the adrenal cortex and increases the cortical concentration of ascorbic acid.[5,11]

The purpose of this work was to confirm the previously reported influence of thyroid-active agents on pituitary-adrenal axis by measuring the variations in the adrenal ascorbic acid concentration and adrenal weight. Moreover, since reserpine and chlorpromazine were reported to possess antithyroidal properties and because these drugs appear to inhibit the effects of heat stress,[14] it seemed desirable to determine the survival rate of rats exposed to high temperatures.

MATERIALS AND METHODS

Five-month-old albino female rats of Sprague-Dawley strain, weighing approximately 250 Gm., were used in all experiments. The animals were maintained under the same conditions on Purina chow and tap water ad libitum.

Methimazole, U.S.P. (1-methyl-2-mercaptoimidazole), was given to rats in drinking water (0.01%; approximately 6 mg./kg./day) for 5 days at room temperature.

Sodium liothyronine, N.N.R. (L-triiodothyronine),* was implanted subcutaneously as a single 1-mg. pellet 10 days prior to the adrenal ascorbic acid determination.

In order to cause heat stress, rats were placed in individual wire restraining tubes in a heat chamber at 43 C. and 54% relative humidity. The body temperature was measured every 15 minutes with

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* Cytomel, supplied by Smith, Kline and French Laboratories, Philadelphia, Pa.

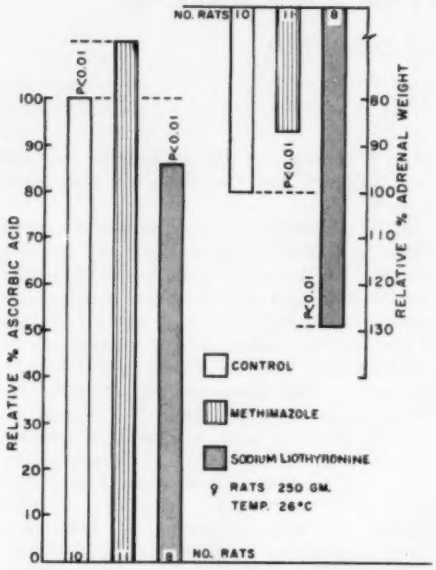

Fig. 1—Effects of 1-methyl-2-mercaptoimidazole (methimazole) and L-triiodothyronine (sodium liothyronine) on adrenal ascorbic acid and adrenal weight in rats at room temperature.

thermocouples inserted rectally to a distance of 3 cm.

Determination of ascorbic acid in adrenal glands was performed spectrophotometrically by the modified method of Roe and Keuther.[17, 18]

PROCEDURE AND RESULTS

Administration of 1-methyl-2-mercaptoimidazole at room temperature increased and L-triiodothyronine decreased adrenal ascorbic acid in rats as compared with the control group (Fig. 1). The opposite effects have been found regarding adrenal weights. As indicated (Table 1), these data were statistically significant.

In the 2nd experiment, rats were premedicated with 1-methyl-2-mercaptoimidazole and, thereafter, they were subjected to a temperature of 43 C. with a constant humidity of 54%. After 75 minutes of exposure, 83% of the treated rats were alive when the 50% survival point was attained by control rats exposed to the same conditions (Fig. 2).

A progressive increase of mean body temperature occurred in rats of both control and treated groups (Fig. 3). The mean body temperature of rats of the latter group

TABLE 1—Effects of 1-methyl-2-mercaptoimidazole (Methimazole) and L-triiodothyronine (Sodium liothyrone) on Adrenal Ascorbic Acid and Adrenal Weight in Rats at Room Temperature

Group	Treatment	No. rats	Adrenal ascorbic acid mg./100 Gm. fresh tissue s.d.	Adrenal weight mg. s.d.
1	Control	10	358.22 ± 6.34	41.56 ± 1.41
2	Methimazole (0.01% in drinking water)	11	404.45 ± 8.51 P<0.01	36.10 ± 1.20 P<0.01
3	Sodium liothyrone (1 mg. s.c.)	8	307.17 ± 12.52 P<0.01	53.80 ± 2.149 P<0.01

P is compared with the control group.

TABLE 2—Effects of 1-methyl-2-mercaptoimidazole (Methimazole) on Rectal Temperature in Rats Exposed to 43 C. and 54% Humidity

Group	Treatment	No. rats	Time of exposure (minutes) 0	30	60	75*
4	Control	12	37.95 ± 0.16	41.17 ± 0.17	42.32 ± 0.15	43.03 ± 0.11
5	Methimazole (0.01% in drinking water)	12	37.86 ± 0.17 P>0.1**	40.79 ± 0.11 P<0.05	41.84 ± 0.11 P<0.05	42.48 ± 0.16 P>0.1

* LD_{50} exposure time for the control rats. ** P is compared with the control group.

TABLE 3—Effects of 1-methyl-2-mercaptoimidazole (Methimazole) on Adrenal Ascorbic Acid, Adrenal Weight, and Survival at 43 C. and 54% Humidity

Group	Treatment	No. rats Used	No. rats Surviving	Adrenal ascorbic acid mg./100 Gm. fresh tissue s.d.	Adrenal weight mg. s.d.	Surviving (%)
4	Control	18	9	200.62 ± 11.55	70.50 ± 2.76	50.00
5	Methimazole (0.01% in drinking water)	18	15	253.00 ± 7.82 P<0.01	69.45 ± 2.17 P>0.1	83.33 P<0.05

P is compared with the control group.

was comparatively lower. However, at the onset and at the end of the heat experiment, the difference was not statistically significant (Table 2).

In spite of the treatment, a very pronounced depletion of adrenal ascorbic acid has been found in all rats surviving heat stress. Although there was not much difference between mean adrenal weights of rats of both groups exposed to heat, the depletion of adrenal ascorbic acid was significantly higher ($P<0.01$) in animals treated with 1-methyl-2-mercaptoimidazole than in the untreated rats (Table 3).

DISCUSSION

The experiments reported here appear to confirm the functional relationship between the thyroid gland and pituitary-adrenal axis. The L-triiodothyronine administered to rats caused some activation of the pituitary-adrenal axis, as indicated by the depletion of adrenal ascorbic acid and the compensatory increase of adrenal weight. An opposite effect has been recorded after blocking the thyroid gland with 1-methyl-2-mercaptoimidazole. This observation is of particular interest for heat stress in animals, because thyroxine and related compounds, as well as cortisone and related compounds, are calorigenic when given in excess. However, the precise mechanism of this activity has not been completely elucidated.

Rats given 1-methyl-2-mercaptoimidazole had increased resistance to heat stress. The survival rate of treated rats was significantly higher than that of controls. Pronounced increase of adrenal weight and depletion of adrenal ascorbic acid due to heat stress were found in both groups of rats. Although there was almost no difference between the adrenal weights of rats in the 2 groups, the adrenal ascorbic acid of rats treated with 1-methyl-2-mercaptoimidazole exceeded that of the controls.

In severe heat stress, when environmental temperature is higher than that of the body, the ordinary mechanism regulating body temperature seems to be somewhat out of order. Therefore, one may wonder whether the action of the thyroid-blocking agent in protecting against heat prostration and the previously reported similar action of chlorpromazine and reserpine [14] are due not only to anti-stress effects but are also, in part at least, due to a direct suppression of body metabolism via the thyroid gland. The mechanism of this activity is still speculative.

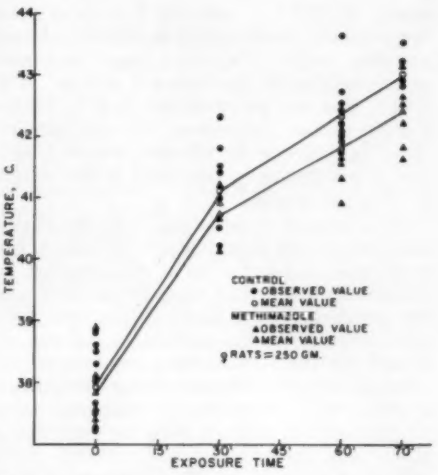

Fig. 2—Effects of 1-methyl-2-mercaptoimidazole (methimazole) on rectal temperatures at 43 C. and 54% humidity.

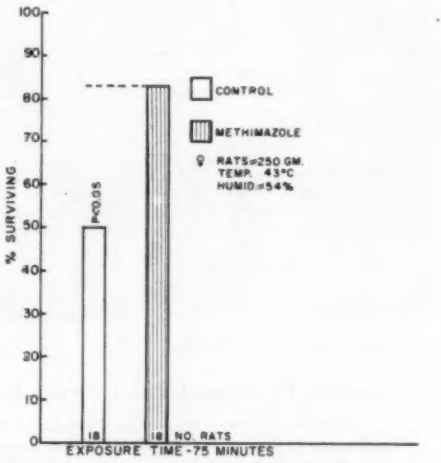

Fig. 3—Effects of 1-methyl-2-mercaptoimidazole (methimazole) on survival.

SUMMARY

An experiment was designed to determine whether thyroid-active compounds influence the survival of rats exposed to high environmental temperatures.

Methimazole, U.S.P. (1-methyl-2-mercaptoimidazole), administered in drinking

water (0.01%) to rats for 5 days at room temperature resulted in increased adrenal ascorbic acid ($P<0.01$) and decreased mean weight of the adrenal glands ($P<0.01$). Sodium liothyronine, N.N.R. (L-triiodothyronine), produced the opposite effect, *i.e.*, decreased adrenal ascorbic acid and simultaneously increased mean weight of adrenal glands.

In a second experiment, rats were premedicated with methimazole as before, but were then subjected to a temperature of 43 C. with a constant humidity of 54%. In the treated group, 83% of the rats were alive when the 50% survival point was attained by the control rats exposed to the same conditions. The surviving treated rats had a lower mean body temperature, a higher level of adrenal ascorbic acid, and a lower but statistically insignificant mean weight of adrenal glands as compared with the control animals.

The mechanism of this activity is still speculative.

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SUMMARIO IN INTERLINGUA

Agentes Thyroido-Active e Stress de Calor in le Ratto

Esseva elaborate un experimento pro determinar si o non agentes thyroido-active exerce un influentia super le longevitate de rattos exponite a alte temperaturas ambiental.

Methimazol (U.S.P.; 1-methyl-2-mercaptoimidazol), administrate in aqua potabile (0,01%) a rattos durante 5 dies a un temperatura normal de interior resultava in un augmento del acido ascorbic adrenal (P minus que 0,01) e in un declino medie del peso del glandulas adrenal (P minus que 0,01). Liothyronina de natrium (N.N.R.; L-triiodothyronina) produceva le effecto contrari, i.e. illo reduceva le acido ascorbic adrenal e simultaneemente augmentava le peso medie del glandulas adrenal.

In un secunde experimento, rattos esseva premedicate con methimazol (como previemente) e subjicite alora a un temperatura de 43 C con un humiditate constante de 54%. In le gruppo tractate, 83% del rattos esseva vive quando le puncto de un superviventia de 50% esseva attingite in le gruppo de controlo in que le rattos esseva subjicite al mesme conditiones. Le supervivente rattos in le gruppo a premedication habeva un plus basse temperatura medie del corpore, un plus alte nivello de acido ascorbic adrenal, e un plus basse (statisticamente non significative) peso medie del glandulas adrenal in comparation con le animales del gruppo de controlo.

Le mechanismo de iste effecto remane speculative.

The Effects of Chlorpromazine on Heat Stress in Pigs

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THE RESULTS on the effects of chlorpromazine, reserpine, and ascorbic acid on resisting heat stress in rats were summarized in a previous report.[14] Under the experimental conditions of that study, chlorpromazine appeared to be more effective than reserpine or ascorbic acid in protecting rats against the effects of severe heat stress. Chlorpromazine protected rats against both the depletion of adrenal ascorbic acid and the increase of adrenal weight as a result of stress by hyperthermia. The present study was undertaken to determine (1) if chlorpromazine influenced the survival rate of pigs exposed to high temperatures and (2) what effects this drug produced on the pituitary-adrenal axis as judged by the adrenal ascorbic acid depletion. It was hoped that the experimental results would be applicable to the clinical problem of hyperthermia in swine, especially in the case of transportation.

MATERIALS AND METHODS

Forty-two pigs, males and females weighing about 27 kg., were used in this experiment. The animals were maintained under similar conditions with respect to diet, humidity, and temperature. Before the experiment, all animals were allotted at random to 4 groups: group 1, 10 control animals to determine the physiologic level of adrenal ascorbic acid in healthy pigs at room temperature (26 C.); group 2, 12 pigs given saline solution intramuscularly and serving as heat-exposed (40 C.) controls; groups 3 and 4, each consisting of 10 heat-exposed pigs given 3 mg./kg. of chlorpromazine intramuscularly. Chlorpromazine, as well as the saline solution, was administered 1 hour prior to the onset of stress.

In order to induce heat stress, pigs were placed in canvas slings in a heat chamber with a constant temperature of 40 C. and a humidity of 54% (Fig. 1).

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Body weight was determined before and immediately after exposure to heat. Body temperature was measured at 15-minute intervals during exposure by thermocouples inserted rectally to a distance of 5 cm. (Fig. 2).

An animal was pronounced dead when respiration ceased (terminal apnea) and the electrocardiogram showed no signs of heart activity (Fig. 3).

Determinations of ascorbic acid in the adrenal glands were performed spectrophotometrically by the modified method of Roe and Keuter.[11, 17, 18] Both glands were excised for determinations immediately after death or electrocution of an animal, and the median parts of each gland (approximately 100 mg. of each) were weighed together, ground in a mortar, and preserved with trichloracetic acid for the spectrophotometric procedure.

Fig. 1—The heat chamber.

It had been established in the preliminary experiments that electrocution produced no detectable effect on the concentration of adrenal ascorbic acid, as compared to decapitation as a method of euthanasia.

PROCEDURE AND RESULTS

Pigs restrained at 40 C. and 54% humidity had almost linear increase of body temperature (Fig. 4). A progressive increase of mean body temperature was found both in pigs given saline solution and in pigs given chlorpromazine, but the temperatures of the latter were significantly lower. However, the difference in temperatures was recorded only in surviving animals. There

TABLE 1—Effects of Chlorpromazine on the Rectal Temperatures of Pigs at 40 C. and 54% Humidity

Treatment	No. pigs	Values	Time									
			0	15′	30′	45′	1 hour	15′	30′	45′	2 hours, 10′	
Control (saline solution)	12	Mean	40.60	41.17	41.62	42.23	42.82	43.26	43.68	44.29	44.57	44.82
		s.d.	0.11	0.13	0.18	0.20	0.23	0.25	0.29	0.32	0.28	0.25
Chlorpromazine (3 mg./kg., i.m.)	20	Mean	39.91	40.30	40.72	41.22	41.64	41.99	42.33	42.65	42.97	43.23
		s.d.	0.06	0.08	0.11	0.11	0.12	0.12	0.13	0.15	0.17	0.19
		P	0.01	0.01	.....	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Two hours and 10 minutes (last column) was the LD_{50} exposure time for control pigs.

was no significant difference between final rectal temperatures of control and tranquilized pigs at the moment of death (45.2 ± 0.24 C.).

Fifty per cent of the medicated pigs exposed to 40 C. and 54% humidity were dead within 2 hours and 10 minutes. This median time of death in the medicated animals was used to present the data of survival of principals exposed to the same conditions (Fig. 5). During the same length of exposure to similar conditions, the survival rate of pigs premedicated with chlorpromazine (3 mg./kg., i.m.) was 90% ($P<0.05$).

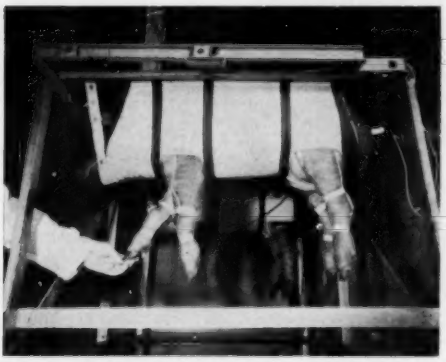

Fig. 2—A pig suspended in a canvas sling in the heat chamber with ECG leads from each leg and thermocouple inserted rectally.

As indicated, 20 animals (2 groups of 10 pigs each) were given chlorpromazine. Members of group 3 were kept in the heat chamber until death. Animals of the other group (4) surviving 2 hours and 10 minutes were electrocuted, *i.e.*, after the median time of death in nonmedicated pigs. The mean time of death in tranquilized pigs (group 3) was much longer (4 hours and 25 minutes) than in untreated pigs (2 hours and 16 minutes).

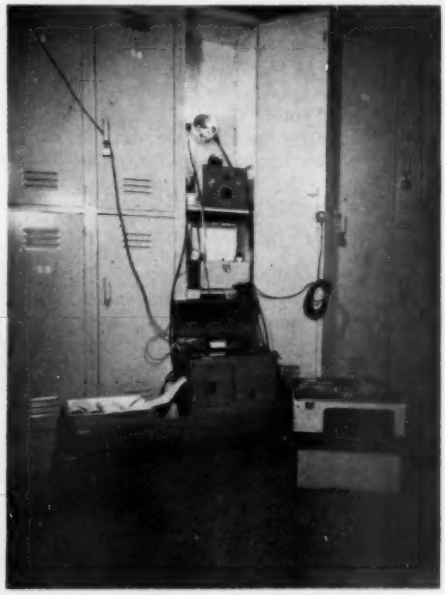

Fig. 3—Apparatus outside the heat chamber for recording rectal temperature, heart action, and respiratory sounds.

Despite pretreatment, heat stress caused pronounced depletion of adrenal ascorbic acid in pigs (Fig. 6). However, the rate of depletion was significantly greater ($P< 0.001$) in nonmedicated animals than in tranquilized ones similarly exposed. There were no significant differences ($P>0.5$) between the concentration of adrenal ascorbic acid in nonmedicated and tranquilized (group 4) pigs at the time of death.

TABLE 2—Effects of Chlorpromazine on Adrenal Ascorbic Acid and Loss of Body Weight in Pigs at 40 C. and 54% Humidity

Group	Treatment	No. pigs	Adrenal ascorbic acid mg./100 Gm. fresh tissue s.d.	Loss of body weight Gm./min. s.d.
1	Control (at 26 C.)	10	251.41 ± 5.39	....
2	Control (saline solution, i.m.; at 40 C. to 100% death)	12	120.34 ± 3.21 $P_1<0.001$	10.15 ± 1.49
3	Chlorpromazine (3 mg./kg., i.m.; at 40 C. to 100% death)	10	118.37 ± 3.20 $P_1<0.001$ $P_2>0.5$	5.50 ± 0.56 $P_2<0.01$
4	Chlorpromazine (3 mg./kg., i.m.; at 40 C. to 50% death)	10	162.53 ± 6.94 $P_1<0.001$ $P_2<0.001$	5.85 ± 0.76 $P_2<0.025$

P_1 = compared with the control group 1; P_2 = compared with the control group 2.

Nonmedicated pigs exposed to an elevated temperature lost much more of their body weight than did pigs given chlorpromazine (Fig. 7). The difference was statistically significant for both groups of pigs treated with chlorpromazine. The results concerning inhibitory effects of chlorpromazine on adrenal ascorbic acid depletion and loss of body weight during heat stress of pigs are summarized (Table 2).

DISCUSSION

Increased ambient temperature stimulated the pituitary-adrenal axis, as indicated by adrenal ascorbic acid depletion. The effects of heat stress in pigs could be diminished with chlorpromazine (3 mg./kg.) injected i.m. 1 hour prior to the onset of stress. Treatment with chlorpromazine significantly increased the survival rate of pigs exposed to elevated temperature. This treatment also resulted in a lower rectal temperature and less depletion of adrenal ascorbic acid. The treated animals experienced less body weight loss. However, the latter data were only of relative value in comparison to the nonmedicated group, because no individual critical metabolic determinations were made.

In 2 pigs, neither tranquilizing nor hypothermic effect of chlorpromazine was observed. The heat-stress reactions of these animals were similar to those of nonmedicated pigs. The animals died within the median time of death for the nonmedicated pigs.

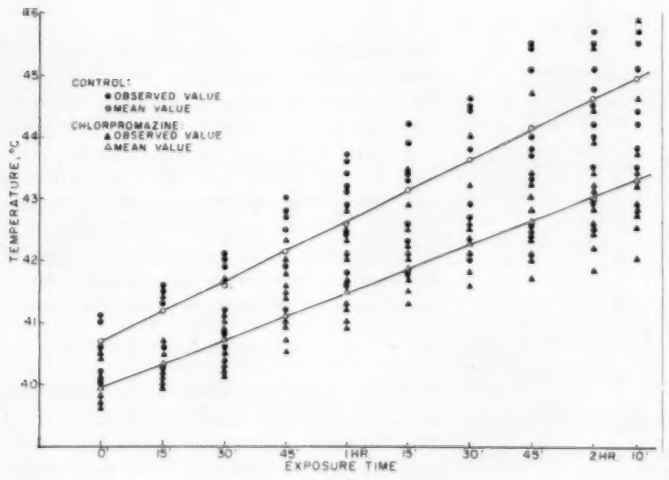

Fig. 4—Effects of chlorpromazine (3 mg./kg., i.m.) on rectal temperatures of pigs at 40 C. and 54% humidity.

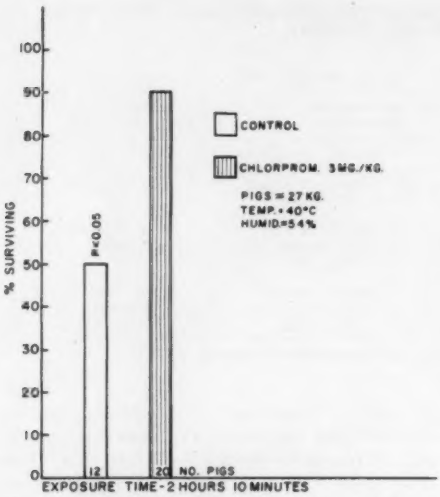

Fig. 5—Effects of chlorpromazine (i.m.) on survival at high temperature.

The results of the present investigation on pigs confirm the results obtained by several other authors who used laboratory animals to demonstrate that chlorpromazine significantly inhibits adrenal ascorbic acid depletion due to stress effects.[9, 12, 15, 19] However, the question of what is the function of the pituitary-adrenal axis in heat stress still seems to be unanswered.

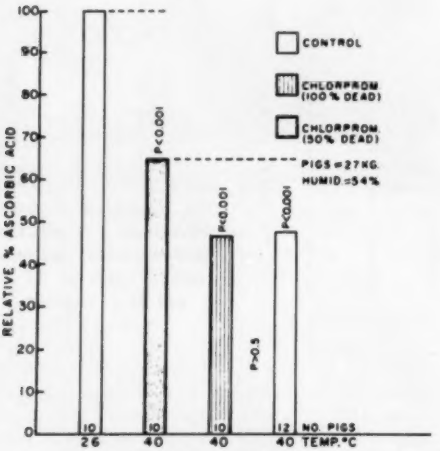

Fig. 6—Effects of chlorpromazine (3 mg./kg., i.m.) on adrenal ascorbic acid.

Copious salivation and panting occurred in both medicated and nonmedicated animals, but the former were slower to show these signs. The medicated pigs also were quiet and less responsive. However, in high relative humidity, with the ambient temperature higher than normal body temperature, the normal mechanism of body temperature regulation could have had little effect.[12, 20]

There are many reports suggesting that chlorpromazine and serotonin produce a comparable decline in oxygen consumption [4, 8, 10] and inhibit adenosine triphosphatase [1, 5, 7, 21] and cytochrome oxidase [4, 6] activity. In addition, marked anti-epinephrine and some anti-histaminic properties of chlorpromazine have been reported.[8, 10, 16] These reports suggest that chlorpromazine may have some influence upon the metabolic rate. If this assumption should be correct, it is in accord with the observation in this study that chlorpromazine decreases the loss of body weight in pigs exposed to hyperthermia. The precise explanation of the mechanism of chlorpromazine effects in acute heat stress has yet to be elucidated.

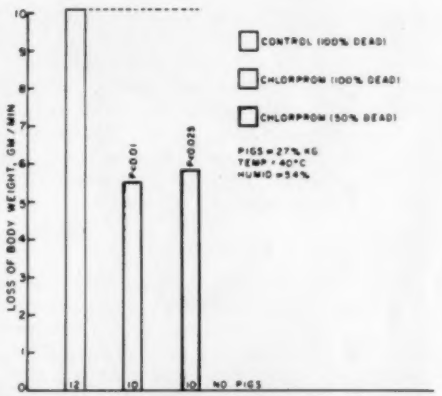

Fig. 7—Effects of chlorpromazine (i.m.) on body weight at high temperature.

SUMMARY

Young male and female pigs, weighing about 27 kg., were used. Fifty per cent of the nonmedicated pigs, exposed at 40 C. and relative humidity of 54%, died within

2 hours and 10 minutes. During an equal exposure time to the same conditions, the survival rate of pigs previously given chlorpromazine intramuscularly (3 mg./kg.) was 90% ($P<0.05$).

In addition, the surviving medicated animals had significantly less loss of body weight. They also had lower mean body temperatures and higher levels of adrenal ascorbic acid as compared to the controls.

These experiments suggest that chlorpromazine diminishes stressful response of the pituitary-adrenal axis in pigs due to hyperthermia, increases the survival rate at 40 C., and decreases the body weight loss.

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SUMMARIO IN INTERLINGUA

Le Effecto de Chlorpromazina Super le Stress de Calor in Porcos

Juvene porcos masculo e feminin con peso de circa 27 kg esseva usate. Cinquanta pro cento del non-medicate porcos moriva intra 2 horas e 10 minutas de exposition a 40 C e un humiditate relative de 54%. Durante le mesme tempore de exposition al mesme conditiones, le superviventia de porcos previemente tractate con chlorpromazina per via intramuscular (3 mg per kg de peso corporee) amontava a 90% (P minus que 0,05).

In plus, le medicate superviventes habeva suffrite un significativemente minus marcate perdita de peso corporee. Illos etiam habeva plus basse temperaturas corporee medie e plus alte nivellos adrenal de acido ascorbic in comparation con le animales de controlo.

Iste experimentos suggere que chlorpromazina reduce le responsa stressose del axe pituitario-adrenal in porcos al effecto de hyperthermia, que illo augmenta le superviventia a 40 C, e que illo reduce le perdita de peso corporee.

Studies on an Immunogenic Agent Against *Brucella abortus*. II. Preliminary Studies of an Immunologically Active Soluble Agglutinogen

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Blacksburg, Virginia

HOAG AND ALLEN [7] reported that a soluble "metabolic by-product" Brucella vaccine, prepared from an acid-heat extracted whole culture, afforded protection in guinea pigs. They further demonstrated that no agglutinin response was elicited at immunologically effective levels.

In the present work, an attempt was made to determine if the agglutinogenic activity of this type of vaccine was correlated with or was distinct from the immunogenic activity.

Acid-heat extracted whole culture and culture-filtrate vaccines, as well as culture filtrate and culture-filtrate fractions prepared electrophoretically, were employed in this study. Since it has been previously demonstrated that the length of incubation of the culture is a determining factor in the success of an immunogenic agent of this type, growth studies were also performed.

MATERIALS AND METHODS

Mediums.—Brucella abortus strain 2308 was grown in Stuart's medium (Difco) to which was added 10% sterile rabit serum.[5, 11] Replicate flasks were seeded with a standard inoculum of this strain obtained from the 2nd medium passage of an isolate from an infected guinea pig spleen. They were incubated at 37.5 C. with periodic shaking. Albimi* agar plates and Albimi agar plates containing 1:50,000 basic fuchsin were used for recovery of the organism from experimentally infected guinea pigs.

Agglutination Test.—The Brucella agglutinin titers of all experimental animals were determined by the standard tube agglutination test of Fitch *et al.*[4] All titers were expressed as the final dilution of the last tube showing agglutination. *Brucella abortus* strain 2308 antigen was prepared after the method of McCullough,[6] except that the organisms were cultured on Albimi agar. Smoothness of antigen cultures was confirmed visually by the use of reflected light [6, 8] and by the saline autoagglutination test.[3] Each lot of antigen was tested and compared with known positive and negative serums.

Growth Studies.—Flasks were removed at 3-day intervals in experiment 1 and at 2-day intervals in experiment 2. Total viable counts for both cultures and challenge material were determined by plating serial dilutions of the cultures on agar plates. Turbidity measurements were made in a spectrophotometer* at 630 mμ against a Stuart's medium blank. Color intensity of the blank and culture, both containing phenol red, was brought to maximum intensity by the addition of a drop of 1.0 normal NaOH prior to reading.

Total protein nitrogen and total nonprotein nitrogen (NPN) were determined, using the micro-Kjeldahl method.

Vaccine Preparation.—Acid-heat extracted vaccines were prepared by heating 14-day cultures to 90 C. and adjusting the pH to 5.6 with 0.1 N acetic acid. The culture was then boiled for 5 minutes, at which time precipitation was visibly complete. The material was cooled and the flocculent precipitate was removed by filtration. The precipitate was washed, dissolved in 0.1 N NaOH, and adjusted to 10 mg. of protein per milliliter.

Cell-free vaccines were prepared from aliquots of the 14-day whole culture passed through a Seitz EK filter. After sterility studies on the filtered material, acid-heat extraction was carried out in a similar manner. Culture-filtrate vaccine and electrophoretic fractions were prepared from a concentrate of the cell-free culture obtained by pervaporation. The cell-free concentrate vaccine was adjusted to a protein concentration of 10 mg. per milliliter, and the aliquot for continuous flow separation was adjusted to 15 mg. of protein per milliliter.

Continuous-Flow Electrophoresis.—Concentrated cell-free culture filtrate was diluted 1:2 with barbital buffer (pH 8.6, ionic strength 0.02) prior to being passed through a continuous-flow electro-

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* Albimi Laboratories, Inc., Brooklyn, N.Y.

* Spectronic Twenty, Bausch & Lomb Co., Rochester, N.Y.

phoresis apparatus.* Separation was carried out at 60 ma., using barbital-barbituric acid buffer (pH 8.6, ionic strength 0.02). Internal temperature was maintained at 20 to 22 C. by circulation of cold buffer through the cooling plate. Phenol red, incorporated into the medium in a final concentration of 0.01%, served as a reference dye by which wick flow and curtain flow could be adjusted prior to fraction collection. The dye later served as a reference point by which approximate Rf values of the fractions could be plotted after staining the curtain with alcoholic bromphenol blue.

Strip Electrophoresis.—Hanging-strip paper electrophoresis in a Durrum-type cell was employed to determine the pattern of the cell-free filtrate, its percentage of the total protein present in the filtrate, and the electrophoretic homogeneity of the fractions collected from the curtain. All materials were run against barbital-barbituric acid (pH 8.6, ionic strength 0.075) at 2.5 ma. for 18 hours on Schleicher and Schuell 3-cm. paper strips. Strips were stained with alcoholic bromphenol blue and read in an integrating scanner.*

Vaccines.—An adjuvant composed of 6% lanolin in mineral oil[5] was added in a ratio of 3 parts adjuvant to 2 parts vaccine (v/v). Prior to addition of the adjuvant, fractions obtained from the curtain were standardized on the basis of each fraction's percentage of the total protein in the cell-free concentrate (Fig. 4), determined by correlating micro-Kjeldahl results with paper strip electrophoretic analysis.

Experimental Procedure.—Experiment 1 was designed to determine if the Brucella cells were necessary to provide protection in a vaccine of this type. Growth studies were made to determine changes taking place during the 14-day incubation period so that an estimation of the optimal point of autolysis could be determined.

Eighty male guinea pigs, weighing 250 to 400 Gm. each, were allotted at random to 20 groups of 4 animals each for a "box-type" titration of various amounts and types of vaccines against various dilutions of challenge material (Fig. 2). Vaccines were inoculated subcutaneously in the cervical region. Serum agglutinin levels were determined prior to vaccination and 28 days postchallenge. Challenge doses were given intraperitoneally.

Twenty additional animals of similar weight and allotment were employed to determine postvaccination titers 18 days following vaccination.

A similar growth study and box-type titration were employed in experiment 2 (Fig. 4) with the following exceptions: (1) Serum agglutinin levels were determined also at 18 days following vaccination. (2) A stronger challenge dose of organisms and less vaccine were used in an attempt to reach an end point in the vaccinated animals. (3) The subcutaneous route of challenge was employed. (4) One dosage level of each type of vaccine preparation was employed (Fig. 3). (5) Agglutinin levels stimulated by each vaccine preparation and killed brucellae were also determined (Table 1) in rabbits. (6) Culture filtrate and its electrophoretic fractions were employed to determine which fraction contained agglutinogenic activity and which contained immunogenic activity.

* Beckman Instruments, Inc., Spinco Division, Belmont, Calif.

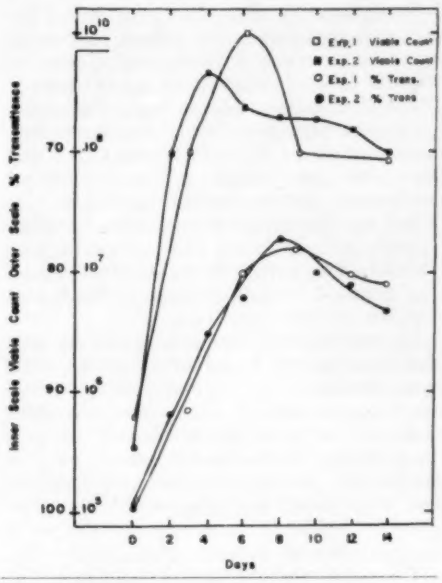

Fig. 1 Growth response curves of Brucella abortus strain 2308 grown in Stuart's medium.

RESULTS

Growth Studies.—Growth response studies (Fig. 1) indicate similar results in both experiments. The maximum viable count was followed by maximum turbidity in about 2 days. In the logarithmic-death phase, autolysis was apparent as indicated by the decreasing turbidity of the cultures (Fig. 1).

Protein and NPN values in the whole culture had no significant quantitative change during the 14-day growth period in either experiment. In experiment 2, however, protein in fraction 1 from the culture filtrate was increased 31% (0.58 mg./ml.). This increase corresponded directly with the decrease in turbidity of the culture and was evident at 12 days. Electrophoretic fractions 2 and 3 underwent no apparent quantitative changes in protein or NPN. For fraction 4, which contained the serum albumin from the medium, there was a decrease of 5% protein (0.3 mg./ml.).

Protection Studies.—The titration of the challenge material in the guinea pigs given no vaccines resulted in an ID_{50} (infective dose for 50% of animals) of approximately 2.5×10^5 organisms per animal. Titrations for guinea pigs given the 2 vaccine preparations gave an ID_{50} of at least 3.1×10^8 organisms per animal, assuming that all vaccinated animals would have been infected by the next lower dilution of challenge material since no end point was reached. These data indicated that protection resulted with both whole-culture and cell-free culture preparations.

Titrations were based on recovery and identification of *Br. abortus* strain 2308 from tissues of animals at necropsy, and not from serologic results. The low postchallenge titers in the vaccinated animals are a further indication of protection. No diminution in the immunologic response was evident by the removal of brucellae prior to acid-heat extraction at the challenge levels and dosages used.

The paper electrophoretic patterns of the cell-free concentrate and its fractions are shown (Fig. 3). Rf values for each fraction, determined against the migration of phenol red, were as follows: fraction 1, 0.096; fraction 2, 0.32; fraction 3, 0.58; and fraction 4, 0.86. The Rf values are expressed as a ratio of the phenol red front to the zone front, measured from a perpendicular line dropped from the point of application to a point just above the serrated edge of the curtain.

Titration results in experiment 2 (Fig. 4) indicated that 50% infection was effected in guinea pigs given no vaccine by approximately 3×10^5 organisms per animal, assuming no infection in the next higher dilution of challenge. Titrations for guinea pigs given the cell-free concentrate and fraction 1 indicated the ID_{50} was 3.1×10^8 and 5.2×10^7 organisms, respectively. Protection, based on greater than a 2-log difference in ID_{50} (vaccinated animals compared to nonvaccinated controls), was demonstrated only in animals of these 2 groups. At the challenge level used, none of the other preparations produced immunogenic activity; however, the acid-heat extracted culture filtrate and fraction 2 resulted in some diminution in the postchallenge titers of treated guinea pigs in comparison to those of the nonvaccinated ones.

Postvaccination titers in rabbits given vaccines without adjuvant are shown (Table 1). It was apparent that both the cell-free concentrate and fraction 1 contained a soluble agglutinogen. The titer elicited by *beta*-propiolactone killed cells, quantitated to the number in the acid-heat extracted whole culture, indicated that acid and heat destroy much of the agglutinogenic activity. However, the higher titer produced by *beta*-propiolactone killed brucellae persisted much longer than that produced by filtrate and acid-heat treated soluble antigens.

DISCUSSION

Postvaccination titers were determined for a separate group of guinea pigs in experiment 1 due to difficulties in obtaining blood samples. Since the primary purpose

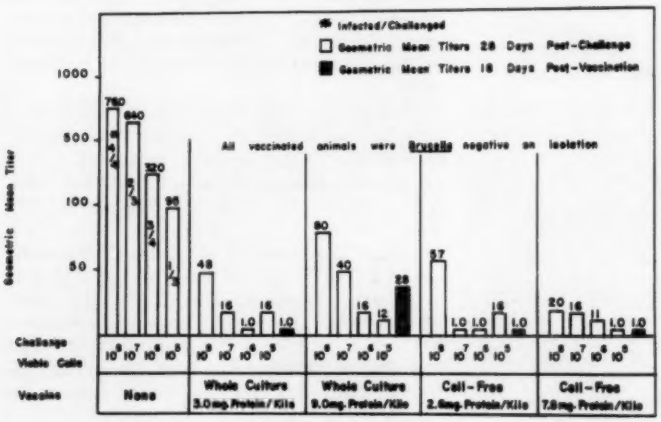

Fig. 2—Geometric mean titers and brucella abortus recoveries from guinea pigs 28 days postchallenge. A separate group of animals was used for postvaccination titers which were determined 18 days postvaccination inoculation. A geometric mean titer of 1.0 indicates no titer at the lowest dilution employed (1:10).

TABLE 1—Agglutinin Response in Rabbits to Various Vaccine Preparations Based on 20 mg. of Soluble Protein in Whole Culture Acid-Heat Extracted Vaccine

Vaccine	Rabbit No.	Postvaccination titer 7 Days	14 Days	21 Days
BPL-killed cells	1	1:2,560	1:2,560	1:1,280
Whole culture A–H (20 mg. protein)	2	1:320	1:80	1:20
	3	1:320	1:40	1:40
Cell-free A–H (20 mg. protein)	4	1:40	1:40	Neg.
	5	1:40	1:20	Neg.
Cell-free concentrate (20 mg. protein)	6	1:80	Neg.	Neg.
	7	1:40	Neg.	Neg.
Fraction 1 (1.26 mg. protein)	8	1:20	Neg.	Neg.
	9	1:80	1:20	Neg.
Fraction 2 (2.2 mg. protein)	10	Neg.	Neg.	Neg.
	11	Neg.	Neg.	Neg.
Fraction 3 (1.4 mg. protein)	12	Neg.	Neg.	Neg.
	13	Neg.	Neg.	Neg.
Fraction 4 (13.2 mg. protein)	14	Neg.	Neg.	Died
	15	Neg.	Neg.	Neg.

BPL = *beta*-Propiolactone. A–H = acid-heat extraction.

of the experiment was to determine if the bacterial cell was a necessary factor in a vaccine of this type, vaccination titers were determined for a separate group of animals only for purposes of supplemental information. With the small number of animals available, excessive deaths due to bleeding by cardiac puncture in the protection experiment would have made this part of the work inconclusive.

The soluble material obtained from the culture during the logarithmic-death phase was agglutinogenic and immunogenic. Continuous-flow electrophoretic separation of the soluble cell-free culture filtrate in experiment 2 was complicated by the presence of rabbit serum in the culture medium. Therefore, it may be stated only that the active fraction migrated in a manner similar to that of *gamma* globulin.

No investigation was made during this study to determine if a similar soluble antigen could be obtained from sonicated extracts of Brucella cells or from cells grown in a serum-free medium. These procedures would alleviate the problem of serum components in the final product, allowing better quantitation and studies of the homogeneity of the active agent found in fraction 1.

It had been determined that agglutinin response and Brucella recovery were both better at 28 days following subcutaneous challenge than intraperitoneal challenge.[1] However, a comparison of the nonvaccinated controls in the 2 experiments at similar challenge levels indicated that there was little difference in the results obtained using the 2 routes of infection.

Vaccination titers and protection studies in experiment 2 indicate that the soluble agglutinogenic fraction (fraction 1) was also immunogenic. Quantitative studies indicated that 6 to 12 μg. of protein material conferred a degree of protection in 250- to 500-Gm. guinea pigs. However, this fraction also included *gamma*-globulin from the medium. This is illustrated in Figure 3 which shows a typical rabbit serum pattern even after 14 days of incubation.

Acid-heat extraction reduced the protective effect of both the whole culture and cell-free culture vaccines at higher levels of challenge. The decrease in activity in fraction 1 over the cell-free concentrate may have been due to several factors. Electrophoretic separation may have caused

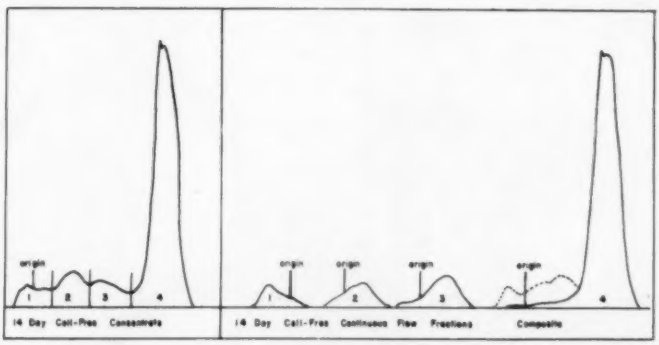

Fig. 3—Paper electrophoresis patterns of Brucella abortus strain 2308 culture filtrate and filtrate fractions obtained from continuous-flow electrophoresis.

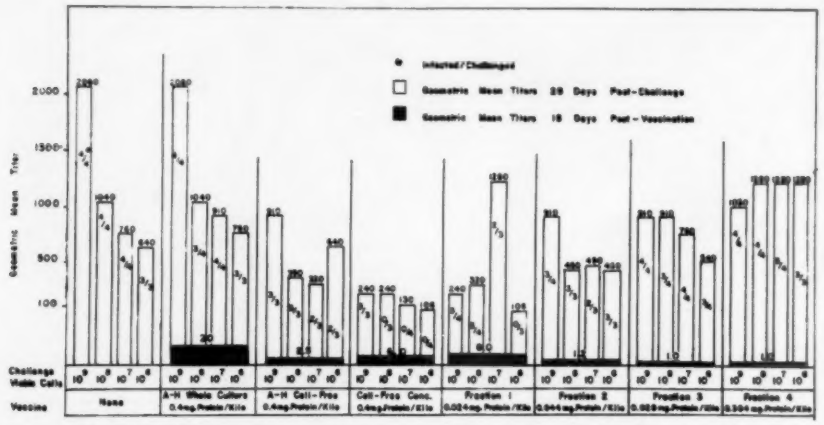

Fig. 4—Brucella abortus recoveries from guinea pigs 28 days postchallenge and geometric mean titers 18 days postvaccination and 28 days postchallenge. A geometric mean titer of 1.0 indicates no titer at the lowest dilution employed.

some denaturation. Some activity may have been carried over into fraction 2, as seen in the reduced postchallenge titer in this group. Finally, the obvious point that too little vaccine was employed must be considered.

This study appears to confirm the view of Sulitzeanu [11] that there are 2 agglutinogens present in brucellae, 1 an insoluble somatic type and the other a soluble type the origin of which is not clear. His view that agglutinating and passive protection powers of serums are due to the same antibody appears to agree with the results obtained in this study. Here, the agglutinogenic and immunogenic properties appeared to characterize the same antigen. However, further work is necessary to determine the source and homogeneity of this antigen before a definite conclusion is warranted.

From the lack of activity in the 2-day culture vaccine reported previously [7] and from the results obtained in this study, it would appear that the role of cell age in the production of this type of material is important. These data suggest that insufficient material was present in the 2-day culture, or that this material may be produced only by older cells in the logarithmic-death phase.

The advantage of a soluble agglutinogen as an immunogenic agent is the fact that the soluble antigen produced low agglutinin titers which were transient in nature. Also, standardization of a soluble material would appear to be simpler than current methods employing attenuated cellular vaccines for the protection against brucellosis.

SUMMARY

A soluble immunogenic antigen against experimental brucellosis in guinea pigs was prepared from the cell-free culture filtrate of *Brucella abortus* strain 2308, grown in a buffered serum medium. The antigen also stimulated low, transient, agglutinin titers in both guinea pigs and rabbits. Data are presented which indicate that the antigen was liberated into the culture medium during the logarithmic-death phase of growth. Further purification by continuous-flow electrophoresis indicated that the antigen migration was similar to that of *gamma*-globulin. Acid-heat treatement of the material diminished, but did not completely destroy, the immunogenic activity.

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SUMMARIO IN INTERLINGUA

Studios Relative a un Agente Immunogene Contra *Brucella abortus*. II. Studios Preliminari Relative a un Immunologicamente Active Agglutinogeno de Character Solubile

Un solubile antigeno de virtute immunogene contra brucellosis experimental in porcos de India esseva preparate ab le filtrato acellular de un cultura de *Brucella abortus*, racia 2308, mantenite in un tamponate medio seral. Le antigeno etiam stimulava basse titros transiente de agglutinina tanto in porcos de India como etiam in conilios. Es presentate datos que indica que le antigeno esseva liberate ad in le medio de culturation durante le phase de morte logarithmic del crescentia. Purification additional per electrophorese a fluxo continue indicava que le antigeno habeva un migration simile a illo de globulina gamma. Le tractamento del material con acido e calor reduceva le activitate immunogene sin abolir lo completemente.

A Heat-Inactivation Test for Differentiating Specific and Nonspecific Agglutination Reactions for Bovine Brucellosis

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MOST SEROLOGIC TESTS for infectious diseases are not completely accurate because of nonspecific reactions. Evidence shows that tests for brucellosis have this limitation. Nonspecific reactions may be particularly responsible for suspect titers and, to a much lesser extent, for minimum reactor titers of the standard tube and plate seroagglutination tests.[5] The purpose of this study was to develop a test for brucellosis that would be capable of differentiating between specific and nonspecific agglutination reactions found in bovine serum. Such a test would be a valuable diagnostic aid in the brucellosis eradication program, especially in determining the status of animals with suspect titers.

The possible use of heat for inactivating nonspecific agglutinins had been recognized earlier. Jones [8] reported that antibody destruction progressed as temperature increased, and that heating at 65 C. for 20 minutes appreciably affected antibody activity. Hoerlein [7] reported that serum of Brucella-free swine, which reacted to the standard tube test, had negative or much lower titers when incubated at 56 C. for 16 hours.

Morse *et al.* [9] obtained similar results using bovine serum. Rose [10] found 2 nonspecific Brucella agglutinins in bovine serum by using physiochemical methods; one agglutinin was heat-stable and the other was heat-labile at 56 C. Hess [6] observed that the nonspecific agglutinins of bovine origin, associated with suspect Brucella titers, were inactivated when heated at 70 C. for 10 minutes.

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In view of the foregoing information, a study was conducted to determine a time and temperature that would eliminate nonspecific reactions in the standard seroagglutination test for brucellosis.

MATERIALS AND METHODS

In the course of this study, 5 experiments were carried out using serum from Brucella-infected and noninfected cattle.*

The reagents used in all experiments were: Brucella tube antigen from strain 1119-3 *Brucella abortus* (4.5% cells by volume) diluted 1:100 with 0.85% NaCl solution containing 0.5% phenol, and freshly prepared 0.85% glucose solution or 0.85% NaCl solution were used for serum diluents before heat treatment and for resuspending heat-treated test samples. Further references to these reagents will indicate that they were used in these concentrations.

Experiment 1.—Fresh, undiluted, bovine serum samples were incubated at 60, 65, 70, 75, 80, 85, or 90 C. for 15 minutes to determine the degree of coagulation produced by heat (in all experiments, incubation was timed after an initial 3-minute warming period). Diluted serum samples were also heated at these temperatures. The serum dilutions were 1:1, 1:2, and 1:3, using NaCl solution or glucose, and 1:25, using antigen.

Experiment 2.—Serum samples were from cattle of known brucellosis status at National Animal Disease Laboratory. Their status had been determined by periodic blood tests and bacteriologic examinations of milk and uterine material at parturition time and afterwards. The effect of heat on serum titers was studied at 56, 60, 65, and 70 C.; each heat treatment was limited to 10, 15, or 20 minutes. Three methods were used.

Method 1.—Serum samples were diluted 1:3 with glucose and heated at the different times and temperatures just described. The agglutination titer was then determined by the standard tube test (STT). This procedure was repeated on each serum sample for each time and temperature.

* The following equipment was adequate: A constant-temperature water bath (tolerance ± 0.2 C.) equipped with motor-driven agitator; a No. 2 International centrifuge, capable of 2,000 r.p.m., with a No. 925 head and No. 926 racks; a Bausch and Lomb Spectronic 20 colorimeter, set at a wave length of 625 mμ with diluted tube antigen used as a blank; Pyrex tubes, 13 by 100 mm.; and a fluorescent titration illuminator (Fisher 11-991-5), black background, for macroscopic reading of test results.

Method 2.—The procedure was identical to that in method 1, except that the mixtures were incubated 15 minutes at 37.5 C. in a water bath instead of 48 hours in a hot-air incubator as in the STT. After incubation, the samples were centrifuged 5 minutes at 1,000 x g (2,000 r.p.m.).[4] After the supernatant fluid was decanted and replaced with 2 ml. of NaCl, the tests were read.

Method 3.—This method, designated as the heat-inactivation test (HIT), was carried out as follows: (1) Dilutions of serum and antigen were identical to the STT, and the antigen was allowed to reach room temperature before use. (2) The serum-antigen mixtures were then immediately placed in a water bath at 65 C. (± 0.2 C.) for 15 minutes; incubation was timed after an initial 3-minute warming period. (3) After incubation, the test samples were removed and immersed immediately in a cold water bath at 18 C. for 3 minutes to terminate the incubation. (4) Test samples were centrifuged 5 minutes at 1,000 x g (2,000 r.p.m.). After the supernatant fluid was decanted and replaced with 2 ml. of NaCl solution, the tests were read. (5) Each tube was shaken gently, and the agglutination was read with the aid of a titration illuminator. Any agglutination in the 1:25 serum-antigen dilution was considered positive and specific. No visible agglutination was considered negative or nonspecific.

At necropsy, internal organs as well as regional lymph glands were cultured for Brucella to establish further the Brucella status of each animal.

Experiment 4.—Examinations were made of individual blood and quarter milk samples collected concurrently from each of 410 dairy cattle in several areas of the United States.[3] The test procedure was the same as in experiment 3. The cattle are identified (Table 3) as naturally exposed.

Blood and milk samples were also collected from 2 government-owned herds at Beltsville, Md. Animals in one Brucella-free herd had never been vaccinated, and animals in the other Brucella-free herd had been vaccinated as calves.

Experiment 5.—The HIT was carried out on an additional 267 serum samples that had been stored at 6 C. for 18 months without a preservative. The test was also made on 532 additional serum samples that had been stored at 6 C. for 7½ months with 1:1,000 thimerosal* added as a preservative. Since the STT had previously been made on the fresh serum samples, the effects of storage on STT titers could also be determined.

RESULTS

Experiment 1.—Heating at 65 C. produced no coagulation in serum diluted with NaCl or glucose solutions (Table 1). However, coagulation was complete after heating undiluted serum at 70 C. or above for 15 minutes. Serum samples diluted 1:1, 1:2, and 1:3 with glucose solution could be heated to 90 C. without complete coagulation, but samples diluted with NaCl solutions in the same ratios coagulated at 75 C. or above. Therefore, either NaCl or glucose could be used to prevent coagulation at 65 C.

TABLE 1—Coagulation of Bovine Serum after 15 Minutes of Incubation at Different Temperatures

Water bath temperature (C.)	Undiluted serum	Serum dilutions with 0.85% NaCl			Serum dilutions with 0.85% glucose			Serum dilution with antigen*
		1:1	1:2	1:3	1:1	1:2	1:3	1:25
60	0	0	0	0	0	0	0	97
65	1	0	0	0	0	0	0	94
70	3	1	1	1	1	1	0	56
75	3	2	2	2	2	1	1	43
80	3	3	2	2	2	1	1	37
85	3	3	3	3	2	1	1	29
90	3	3	3	3	2	1	1	20

0 = no visible coagulation; 1 = slight coagulation; 2 = moderate coagulation; 3 = complete coagulation (material is solid and the tube may be inverted without loss of liquid).
* Per cent of light transmission.

Experiment 3.—Blood and milk samples were obtained from 143 cattle artificially exposed to Brucella at the National Animal Disease Laboratory. The milk samples were collected aseptically from each quarter, cultured on modified tryptose agar, and inoculated into guinea pigs for bacteriologic examination according to the method of Goode *et al.*[2] Blood samples were centrifuged, and the serum was removed. Each serum was immediately tested by the STT as well as by the HIT. The STT results were classified according to the new interpretation,[1] and the HIT results were interpreted according to the method used in experiment 2. A sample of uterine contents, collected at parturition, was examined for Brucella organisms.[2]

The effect of various temperatures on the coagulation of serum diluted 1:25 with

* Merthiolate, Eli Lilly & Co., Indianapolis, Ind.

antigen is also shown (Table 1). The numerical readings determined with the colorimeter represent percentage of light transmission at a wave length of 625 mμ after the serum-antigen mixtures had been heated. The percentage of light transmission at any given temperature is associated with the degree of coagulation produced by the heat treatment of serum. There was no significant difference in the degree of light transmitted by antigen-serum mixtures which were unheated and those that were heated at 60 and 65 C. No measurable difference was detected at any temperature to which the tube antigen alone was subjected. The first interference of light transmission in the serum-antigen mixtures was observed at 70 C.; therefore, the diluted tube antigen was also capable of preventing serum coagulation at 65 C. Thus, it is evident from the methods employed that 65 C. is the maximum temperature at which diluted serum can be heated without causing some degree of coagulation.

Experiment 2.—The most effective and practical time and temperature found for inhibiting nonspecific reactions and retaining specific reactions were 15 minutes and 65 C. Serum samples heated to temperatures below 65 C. required longer incubation periods to remove nonspecific agglutination reactions. These incubation periods were progressively longer with each decrease in temperature. Incubation of serum at temperatures above 65 C. for 10 minutes or longer not only removed all nonspecific reactions in the serum samples studied, but also lowered or completely removed specific reactions in most instances.

The titers with the STT and HIT in serum from cattle of known brucellosis status are shown (Table 2). The 3 methods previously described were used in heat treatment. Test readings were higher with methods 2 and 3 with centrifugation than with method 1, where particles were allowed to settle out of solution. The lower readings with method 1 were associated with smaller particle size and incomplete settling. Because of the difficulty of reading the STT in method 1, and the additional step required in method 2, method 3 was selected to evaluate further the effect of heat on the stability of specific and nonspecific agglutination reactions.

TABLE 2—Effect of Heat Treatment on Agglutinins in Serums of Cattle with Known Brucellosis Status

Brucellosis status	Standard tube titer of serum	Titer after 65 C. heat treatment for 15 minutes		
		Method 1	Method 2	Method 3
Noninfected Nonvaccinated	−25	−25	−25	−25
Noninfected Vaccinated	I 50	−25	I 25	−25
Infected Nonvaccinated	+1,600	I 800	I 3,200	I 3,200
Infected Vaccinated	+200	I 100	+200	+200

− = no agglutination; I = incomplete agglutination; + = complete agglutination.

Prozones encountered in testing serum samples were not completely removed by the procedure of centrifugation and resuspension in saline solution; however, they were only encountered in several serum samples with titers of +1:800 or higher. As these prozones were not present when the samples were tested by the STT, they were considered to be caused by the heat-inactivation procedure.

The method of reading the agglutination reactions produced by the HIT was for all practical purposes the same as that used in the STT. Centrifugation packed all of the agglutinated particles in the bottoms of the tubes, and resuspension in a clear fluid (0.85% NaCl) made them easily detectable. Occasionally, the agglutinated particles were found tightly adhering to the bottoms of the test tubes. This film of agglutinated particles could not be dislodged even after vigorous shaking. Since this type of reaction was encountered only in serum from Brucella-infected cattle, such tests were considered positive. The procedure for reading negative test results presented no problems. The unagglutinated bacterial antigen produced a homogeneous cloud when shaken, which was identical to negative reactions found when samples were tested by the STT. Grossly contaminated or hemolyzed serum samples produced a stringy, mucoid type of agglutinated particle. Results from such samples were not included in the present report because they were of no diagnostic value.

Experiment 3.—Results of the STT and HIT on 143 serum samples obtained from artificially exposed animals are presented (Table 3).

The HIT results from vaccinated cattle were positive for 2.2% of the STT-negative serum samples, 71% of the suspect samples, and 100% of the reactor samples. The 2.2% of the STT-negative serum samples that were HIT positive consisted of 2 ani-

mals from which Brucella was isolated and which had STT titers of I 1:50 and +1:50.* Thirty days later, both animals had STT reactor titers. The 71% of the STT-suspect cattle that were HIT positive consisted of 10 animals. Brucella was isolated from 8 of these 10. Of the 8 animals, 6 later had reactor titers, while 2 remained suspect. Brucella was never isolated from the 2 other suspect animals, although their titers later reached the reactor level. The 4 animals that had STT-suspect titers, but whose HIT titers were negative, never reached the STT reactor level nor was Brucella ever isolated. The 100% of the STT reactor cattle that were HIT-positive consisted of 12 animals. Brucella was isolated from all 12.

Serum samples from 4 of 28 nonvaccinated artificially exposed cattle were HIT and STT negative. Isolations of Brucella were made from the blood of 1 animal on 2 consecutive blood samplings, 2 months after exposure, at the same time the agglutination test results were obtained. This animal's titer never reached the suspect or reactor level nor were any further Brucella isolations made at the time of parturition or necropsy. However, this animal was not classified as having developed brucellosis, since infection was based on the criteria established by members of the Brucellosis Research Conference.[2] Occasionally, cattle exposed to virulent *Brucella abortus* at the level employed in these studies develop a transient bacteremia or seroagglutinins following the exposure yet do not develop the disease. Only 2 nonvaccinated animals with STT-suspect serums were encountered. Serum from these animals was positive to the HIT, and Brucella was isolated from both. One animal's serum was tested by the STT and the HIT on the day the animal aborted. The other animal's serum was not obtained for testing until 6 days after abortion. In both instances the HIT test was positive and the STT was +50, but later the serum titers of each animal reached a high reactor level. Serum samples from 22 bacteriologically positive STT-reactor cattle were also HIT positive.

Experiment 4.—Results of the STT and HIT on 410 serum samples obtained from naturally exposed vaccinated and nonvaccinated cattle are presented (Table 3). Only 9 of 249 vaccinated cattle which were STT negative were positive to the HIT. Seven of these were from multiple-reactor herds and 2 from suspect herds. The serum samples of 21 of 84 vaccinated cows with suspect titers were HIT positive. Of the 21, 16 serum samples were from cattle in multiple reactor herds, and 5 were from suspect herds. Brucella was isolated from the milk of 1 of the 16. All 11 vaccinated cattle with reactor titers were from multiple-reactor herds. Seven of these were HIT positive, with isolations of Brucella from 4. Brucella was not isolated from the remaining 4 whose serum was negative to the HIT.

Of the serum tested from the nonvaccinated cattle, 2 of 33 STT-negative samples were HIT positive. Both samples were from animals in multiple-reactor herds. Eight of 17 STT-suspect samples were HIT positive. Six of these were from multiple reactor herds and 2 from suspect herds. Of the 9 HIT-negative samples, 5 were from suspect herds, 3 from single-reactor herds, and the other from a multiple-reactor herd. No Brucella was isolated from the STT-negative or suspect cattle. Twelve of the 16 STT-reactor samples were HIT positive. Ten of these 12 were from multiple-reactor herds, and Brucella was isolated from 2. The remaining 2 herds each contained 1 reactor animal, and no Brucella was isolated. Brucella was not isolated from the milk of the 4 HIT-negative cattle, even though they were from multiple-reactor herds.

During the course of these studies, 10 animals of 150 in the Brucella-free herd at the National Animal Disease Laboratory developed titers to the STT for brucellosis. Nine of the 10 animals had suspect titers and 1 had a reactor titer that never ex-

TABLE 3—Results of Studies Employing a Heat-Inactivation Test for Detecting Specific Brucella Agglutination Reactions

Classification by STT	Vaccinated cattle: Serums tested (No.)	Vaccinated cattle: HIT Pos.	Vaccinated cattle: HIT Neg.	Nonvaccinated cattle: Serums tested (No.)	Nonvaccinated cattle: HIT Pos.	Nonvaccinated cattle: HIT Neg.
EXPERIMENT 3—ARTIFICIALLY EXPOSED CATTLE						
Negative	89	2(2)	87	4	0	4
Suspect	14	10(8)	4	2	2(2)	0
Reactor	12	12(12)	0	22	22(22)	0
Total	115	.....	....	28	.....	..
EXPERIMENT 4—NATURALLY EXPOSED CATTLE						
Negative	249	9	240	33	2	31
Suspect	84	21(1)	63	17	8	9
Reactor	11	7(4)	4	16	12(2)	4
Total	344	.....	....	66	.....	..

Numbers in parentheses indicate number of cattle from which Brucella was isolated from the milk or uterine material, or tissues at necropsy; STT = standard tube agglutination test; HIT = heat-inactivation test.

* I = incomplete agglutination; + = complete agglutination; − = no agglutination.

ceeded I 1:200. Results of the HIT were negative for all 10 animals. All 9 suspect animals returned to a STT-negative status within a few weeks. The reactor animal was removed from the herd for further study. Brucella was never isolated from the milk or genital tracts of any of the cows.

The serum samples of 10 calf-vaccinated animals from another government-owned herd, containing cattle with suspect titers, were subjected to the HIT. All of these samples were HIT negative except 1. A single milk collection from the animal failed to disclose the presence of Brucella, and the animal was unavailable for further study. The herd from which these cattle originated has been under study since 1940, and the last time Brucella was isolated was in 1950. This isolation was made from the milk of the last persistent reactor found in the herd.

Experiment 5.—Retests of the 267 serum samples stored at 6 C. for 18 months without a preservative were made by the STT method. It was found that 13% of the samples had decreased in titer by 2 or more dilutions, 34% by 1 dilution, and 51% remained unchanged, while 2% were 1 dilution higher. Retests of the 532 additional serum samples by the STT after 7½ months of storage at 6 C. with 1:1,000 thimerosal added as a preservative were as follows: 2% had decreased in titer by 2 or more dilutions, 18% by 1 dilution, and 79% remained unchanged, while 1% were a dilution higher. The HIT results of both groups of stored samples were extremely erratic when correlated with bacteriologic evidence and with herd histories. The STT and the HIT have little or no value when conducted on serum stored under the conditions of this experiment.

Data compiled from the reported experiments have been divided into 2 major groups, that obtained from vaccinated cattle and that obtained from nonvaccinated ones. The data were reported separately for each group because of the titer differences between negative, suspect, and reactor classification for vaccinated cattle as opposed to the titer level used for the same classification of nonvaccinated cattle. The other reason for reporting the results in this manner was to determine and compare the percentage of HIT-positive samples from vaccinated cattle to HIT-positive samples from nonvaccinated cattle. Calculations of these percentages from the data of the vaccinated group (Table 3) show that 3, 32, and 83% of the negative, suspect, and reactor cattle, respectively, had serum samples that were HIT positive. In comparison, 5, 53, 89% of the nonvaccinated cattle in the negative, suspect, and reactor categories, respectively, had serum that was HIT positive.

DISCUSSION

Since the STT is recognized as a standard serologic procedure, and as such has been an invaluable tool in the control and eradication of bovine brucellosis, the HIT has not been offered as a substitute. The HIT was designed only as an aid in testing STT-suspect serum to determine the specificity of the agglutinins present. If the HIT is employed in this manner, 68% of the vaccinated animals and 47% of the nonvaccinated animals that had suspect titers to the STT in experiments 3 and 4, respectively, could be classified as Brucella-free. Thus, the suspect problem could be decreased without the probability of classifying infected animals as negative. Further evidence of the accuracy of the HIT has been obtained from experiment 3. Here it was shown that, under controlled conditions and extensive bacteriologic examination, Brucella could be isolated from 96% of the cattle that had serum positive to the HIT. Thus, from the data presented, it is improbable that infected suspect animals, having specific Brucella agglutinins in their serum, would be undetected by the HIT.

It would appear that if the bacteriologic examinations prevailing in experiment 3 could have been duplicated in experiment 4 (naturally exposed cattle), many more isolations of Brucella would have been made from cattle with serum samples that were positive to the HIT. Even so, when the HIT was applied to serum from STT-suspect cattle in the various types of herds, no infected cattle were classified negative by the HIT. Thus, the evidence suggests that it is possible to distinguish between specific and nonspecific Brucella agglutinins by means of the HIT.

The HIT-positive results obtained from STT-suspect cattle that were bacteriologically negative also are worthy of consideration. Twenty such vaccinated and 8 nonvaccinated cattle in experiment 4 were all from herds containing 1 or more reactors with the exception of 5 vaccinated and 2

nonvaccinated cattle that were from suspect herds. The fact that these serum samples were positive to the HIT does not necessarily mean that the test was in error. The evidence developed suggests that animals whose serum samples react positively to the HIT have received specific stimulation with Brucella. Similarly, it was demonstrated that suspect cattle in a known Brucella-free herd did not react to the HIT.

In the authors' opinion, the HIT could be used (1) to clarify the status of suspect herds when there is no other evidence of brucellosis and (2) to decrease the degree of exposure in an infected herd by removing HIT-positive suspects.

SUMMARY

Studies were conducted on the thermolability of specific and nonspecific agglutinins for brucellosis found in bovine serum. A heat-inactivation test (HIT) was devised to inactivate nonspecific seroagglutinins encountered in the standard tube agglutination test (STT) for brucellosis

Serum samples from 143 artificially exposed cattle and 410 naturally exposed cattle were tested by the standard tube agglutination test and the heat-inactivation test with the following results:

1) Positive HIT serologic reactions were obtained from all of the artificially and naturally exposed STT-suspect and reactor cattle from which *Brucella abortus* was isolated.

2) Negative serologic reactions were obtained from 74% of the artificially and naturally exposed STT-suspect cattle from which *Brucella abortus* was not isolated.

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SUMMARIO IN INTERLINGUA

Un Test de Thermo-Inactivation pro le Differentiation Inter Specific e Non-Specific Reactiones Agglutinatori de Brucellosis Bovin

Esseva effectuate studios in re le thermolabilitate de agglutininas specific e non-specific pro brucellosis que es trovate in seros bovin. Un test de thermo-inactivation (TTI) esseva elaborate pro inactivar non-specific seroagglutininas incontrate in le test agglutinatori standard a tubos (TST) pro brucellosis.

Specimens de sero ab 143 artificialmente exponite pecias de bestial e ab 410 naturalmente exponite pecias de bestial esseva testate per le TST e le TTI con le sequente resultatos:

1. Positive reactiones serologic in le TTI esseva obtenite ab omne le artificialmente e naturalmente exponite animales que esseva reactores o suspecte in le TST e ab que *Brucella abortus* esseva isolate.

2. Negative reactiones serologic in le TTI esseva obtenite ab 74 pro cento del artificialmente e naturalmente exponite animales que esseva suspecte in le TST e ab que *Br. abortus* non esseva isolate.

Fluorescein-Labeled Antibody Technique for Identification of Leptospiral Serotypes

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IDENTIFICATION of leptospiral serotypes is a tedious and lengthy procedure, requiring the production of large quantities of the organism to be identified, the production of antibodies against these organisms, and the subsequent cross-absorption studies.

The fluorescein-labeled antibody technique has been applied to the staining of several leptospiral serotypes. Moulton and Howarth [1] demonstrated that they could stain *Leptospiral canicola* in hamster kidneys. Sheldon [2] described the staining of *Leptospira icterohaemorrhagiae* in the gastrocnemius muscle of a human patient affected with Weil's disease. White and Ristic [3] demonstrated staining of *Leptospira pomona* in guinea pig tissues and bovine urine. They used a formalin-fixation technique for the leptospires, and found that, in addition to staining *L. pomona* with *pomona*-labeled hyperimmune serum, they were also able to stain *L. canicola*, *L. icterohaemorrhagiae*, and *Leptospira sejroe* with the same labeled serum. This report describes a definitive method for the identification of leptospiral serotypes by the fluorescent antibody technique.

MATERIALS AND METHODS

Preparation of Antiserum.—The following 10 Leptospira serotypes were used for immunizing rabbits: *L. autumnalis*, *L. australis*, *L. ballum*, *L. bataviae*, *L. canicola*, *L. icterohaemorrhagiae*, *L. mini*, *georgia*, (LT117), *L. pomona*, *L. pyrogenes*, and *L. sejroe*. The organisms were grown in Stuart's medium for 4 to 6 days at 28 C., with good heavy cultures being obtained usually by the 4th day. Live organisms were used for the immunization and a series of 5 injections was administered at weekly intervals. The first inoculation of 1 ml. was given subcutaneously. The other 4 inoculations were given intravenously in the amounts of 1, 2, 4, and 6 ml. on successive weeks. Seven days after the last injection, blood samples were obtained from the hearts of the rabbits, the cells discarded, and the globulin separated by precipitation with half-saturated ammonium sulfate. Precipitated globulins were then dialyzed against physiologic saline solution to remove the ammonium sulfate.

Labeling of Globulin.—Globulin was labeled with fluorescein isothiocyanate by the addition of the dye in powder form to a 2.5% solution of globulin in physiologic sodium chloride containing 10.0% carbonate-bicarbonate buffer (0.5 M, pH 9). The dye was added in 4 increments, and conjugation was allowed to proceed for 6 hours following the last addition. The sample was dialyzed in buffered NaCl solution for approximately 48 hours to remove the unreacted dye. Conjugated globulin was not absorbed with tissue powder. Dilutions referred to in the results were dilutions of the original 2.5% conjugated protein solutions.

Preparation of the Organisms for Staining.—The organisms stained with these conjugates were grown in Stuart's medium for 4 to 6 days at 28 C. A small drop of the leptospiral suspension was placed on a microscope slide, and the slide was inverted over a 25-cc. beaker containing 3 to 4 ml. of a 1 to 2% osmic acid solution. The organisms were fixed by the osmic acid vapors. The time of osmic acid vapor fixation was varied from 5 to 60 seconds, depending upon the freshness of the osmic acid solution, the concentration of the solution, and the distance of the slide from the surface of the solution. Optimum fixation time was determined for each batch of slides to be used. The drop of fixed leptospires was spread over a small area and allowed to air dry.

Use of osmic acid solution in a laboratory containing vapors of various organic solvents was not as efficient as when the osmic acid solution was used in the absence of such vapors. The osmic acid vapors tended to react with the organic vapors present in the air. It was found that osmic acid was readily reduced and, as reduction proceeded, the solution became blackened. When the discoloration of the solution was marked, the osmic acid was no longer used as a fixative. The solution stored in sealed ampules without a vacuum became reduced within several weeks; however, storage of the solution in a vacuum at −10 C. has proved satisfactory over a period of several months.

Titration of Labeled Serum.—Serums to be titrated were diluted with veronal buffer. Labeled serums were diluted tenfold from 1:10 through 1:130.

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Staining and Examination of Smears.—The fixed smears, with the fluorescein-labeled antibody on them, were placed in a wet chamber and incubated for 1 hour at 37 C. Following a rinse with buffered NaCl solution, the smears were immersed in a similar solution for 10 minutes, then removed from the saline solution and allowed to air dry. When the smears were completely dry, glycerin mounting medium and a cover slip were placed on them and they were examined with a Reichert dark-field microscope, using a Reichert HBO-200 ultraviolet light. The filter system consisted of a Wratten 2A eye-protection filter in the ocular lens and a Reichert No. 5850 UV pass filter.

The fluorescence of the stained leptospires was graded on a 4+, 3+, 2+, 1+ basis; 4+ indicated marked brilliance of stain, 3+, a similar intensity but not as brilliant a stain, 2+ indicated the organisms were plainly visible but lacking in brilliance, 1+ indicated a stain that was just distinctly visible, and ± indicated leptospires that were very difficult to see; the 1+ titers were taken as the end point.

powder to remove nonspecific staining proved unnecessary, since the conjugates used in the test were diluted beyond the point of nonspecific fluorescence.

DISCUSSION

Leptospires fixed with formalin prior to staining with fluorescein-labeled antibody undergo a great degree of cross staining, thereby limiting the use of this technique to generic identification. The tedium involved in the serologic identification of leptospires by conventional laboratory methods makes the definitive identification of these organisms both laborious and expensive. The task can be performed in less time and at less expense by use of osmic acid fixation on the leptospires. Because of the harsh nature of osmic acid fixation, many leptospires were found to be destroyed by this process. Consequently, this limits the use of osmic acid fixation to a taxonomic study in which adequate numbers of organisms are present. It appears possible that this technique has application to the identification of leptospiral serotypes found in tissue sections, provided the sections were frozen and fixed with osmic acid vapor.

When comparing time intervals of fixation, 5 to 10 seconds gave adequate fixation of leptospires, provided a fresh 2% solution was used and the slide was approximately ¼ inch above the solution. The number of organisms rapidly diminished with increasing time of exposure, and at 30 seconds there were no leptospires found.

With age, the fixative loses strength and

TABLE 1—Cross Titers of Leptospires Fixed with Osmic Acid Vapor and Stained with Fluorescein-Labeled Antibodies

	Serums									
Organisms	*australis*	*autumnalis*	*ballum*	*bataviae*	*canicola*	*icterohaemorrhagiae*	*mini*	*pomona*	*pyrogenes*	*sejroe*
australis	1:80	1:10	1:30	neg.	neg.	neg.	neg.	neg.	1:30	neg.
autumnalis	1:10	1:80	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
ballum	neg.	neg.	1:70	1:10	neg.	neg.	neg.	neg.	neg.	neg.
bataviae	neg.	neg.	1:10	1:70	1:10	neg.	neg.	neg.	neg.	neg.
canicola	1:10	1:10	1:10	neg.	1:40	neg.	neg.	neg.	neg.	neg.
icterohaemorrhagiae	neg.	neg.	neg.	neg.	neg.	1:90	neg.	neg.	neg.	neg.
mini	neg.	neg.	neg.	neg.	neg.	neg.	1:120	neg.	neg.	neg.
pomona	neg.	neg.	1:30	neg.	neg.	neg.	neg.	1:90	neg.	neg.
pyrogenes	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	1:80	neg.
sejroe	neg.	neg.	neg.	neg.	neg.	neg.	1:10	neg.	neg.	1:40

RESULTS

Homologous titers (Table 1) were *L. mini, georgia* (LT 117), 1:120; *L. icterohaemorrhagiae* and *L. pomona*, 1:90; *L. autumnalis* and *L. australis*, 1:80; *L. ballum* and *L. bataviae*, 1:70; *L. pyrogenes*, 1:80; *L. sejroe* and *L. canicola*, 1:40. Cross staining of the various organisms was carried out with a 1:10 dilution of the original conjugated material. It may be seen (Table 1) that cross reactions were few and occurred only in low dilutions of the conjugate. The few low-titer cross reactions in no way interfered with the identification of specific serotypes.

The absorption of conjugate with tissue

it becomes necessary to increase the exposure of the organisms to the fixative either by lengthening the time or moving the slide closer to the surface of the fixative. Therefore, in order to use this method of fixation with facility, it is important that the degree of fixation be tested for each series of slides that is prepared. The degree of fixation of the leptospires in no way affected the titer of the labeled serum.

SUMMARY

Fluorescein-labeled antibodies were found to stain Leptospira sp. specifically after the organisms had been fixed with osmic acid vapor, a harsh fixative that destroys many organisms and deteriorates with age.

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SUMMARIO IN INTERLINGUA

Le Technica del Marcation Fluoresceinic de Anticorpore in le Identification de Serotypos Leptospiral

Esseva trovate que anticorpores a marcation fluoresceinic effectuava un tincturation specific de Leptospira sp. post que le organismos habeva essite fixate con vapor de acido osmic. Isto es un fixativo brutal que destrue multe organismos e que se deteriora con le tempore.

The Detection of Antibody Against *Leptospira pomona* in Composite Herd Milk Samples

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THE ECONOMIC importance of leptospirosis to the livestock industry has been well established since it was first reported by Jungherr in 1944.[6] Needless to say, leptospirosis has reached the stage where some thought and effort should be made to develop a suitable control program. It would appear that the prevalence of *Leptospira pomona* infection in cattle and swine has not been due to wildlife reservoirs alone. Considerable transmission of infection among cattle must be conceded. Other serotypes are important in different countries of the world and their presence in the wild animal species of this country is a cause for concern. However, it has been stated that *L. pomona* was the agent in 98% of the epizootics of leptospirosis in swine and cattle.[7]

The value of the brucellosis milk ring test in locating Brucella infection suggested the development of a similar technique for the diagnosis of leptospirosis. Attempts were made to stain suspensions of leptospires and prepare antigens which would produce a typical milk ring reaction. It was found that effective staining of the leptospires with the technique used in preparing the brucellosis ring test antigen completely destroyed the antigenicity of the cells.[1] Other stained leptospiral antigens also had greatly reduced antigenicity. When these stained suspensions of leptospires were placed in negative milk and the cream allowed to rise, excellent rings were produced. It was questioned whether the physical nature of the leptospires would permit the cells to escape the sieving action of the fat globules even though no agglutination had taken place.

Attention was turned to the possibility that antibody levels in a herd milk sample, taken from either bulk tank or milk can, might be too low to permit detection. If this were true, herds infected with leptospirosis could not be located in this manner. Since the agglutination-lysis test was the most sensitive serologic technique, it was decided to employ it on herd milk samples. The ability of this test to detect leptospiral antibody in bulk tank or milk can samples might lead to its successful application as a convenient screening test for the location of herds infected with leptospirosis. The project described here was intended to determine the feasibility of this procedure and, if a measurable amount of antibody was present, to compare the titer of the herd milk sample with the serum titers found in the individual cattle.

LITERATURE

The presence of specific antibody substances in milk has been recognized for some time. In 1893, the German chemists, Brieger and Ehrlich,[2] succeeded in separating an "antitoxin" from the milk of a goat which had been immunized by repeated injections of the toxic products of the tetanus bacillus. A chemical process was used to extract a fraction from the milk which was 400 to 600 times as effective in neutralizing the tetanus toxin as the milk itself.

In 1915, Seddon,[5] in Australia, attempted to extend the application of the agglutination test for brucellosis to the milk of cattle but found the diluted milk unsuitable because of its physical properties. It was found that, if the whey were separated from the milk, a satisfactory test could be performed. The whey was prepared by mixing 9 parts of milk with 1 part of a 10% aqueous solution of lactic acid. The coagulated milk was removed with filter paper. Serial dilutions of the whey were prepared in phenolized NaCl solution and an "emulsion" of Brucella cells was added. It was found that the agglutinins in the whey increased and decreased in the same manner as those in the blood serum.

Cooledge, in 1916, employed both milk and "rennet milk serum" as means of indicating the presence of infection with *Bacterium abortus*.[4] Complement-fixation and tube-agglutination tests were utilized. It was found that the inoculation of a 48-hour culture into the milk cistern of a cow caused the appearance of agglutinins in the milk. The milk test was considered of diagnostic value since agglutinins were always present in the milk when the organism was isolated.

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TABLE 1—The Results of Whey Agglutination-Lysis Tests on Bulk Tank Milk Samples from 30 Dairy Herds Located near Ames, Iowa

Herd	Whey titer* April	Whey titer* October
W & M	32**	8
CDT	16	8
KH	32	16
DFA	N†	64
FLM	N	16
AS	N	2
CD	N	4
FF	2	2
CEW	4	N
HF	2	N
JB	2	N
DF	2	4
CDF, PT, AHD, HL, RSS, BF, CR, EM, LCA, ON, FS, ORB, MA, CM, HO, AR, LD, RC	N	N

* Twofold dilution scheme (1:2, 1:4, 1:8, etc.). ** Reciprocal of end point titer. † Negative at the 1:2 dilution.

The relation of the blood serum titer to the milk whey titer was further studied by Smith *et al.*[10] In cows inoculated subcutaneously or intravenously with killed suspensions of Brucella cells, the ratio of antibody content in the milk to that in the blood was found to be 1:32 and 1:64. However, when the udder was invaded or made the site of multiplication by *Br. abortus*, the whey agglutinins rose to a new ratio of 1:4, 1:2, and even 1:1.

In 1952, van der Hoeden[13] called attention to the large amounts of agglutinin present in the milk of cows infected with leptospirosis. Although the milk was occasionally negative in the early stages of the disease, positive agglutination tests from milk were always specific. A case of leptospirosis in a goat was described where the milk titer was 1:4,000 at the same time the blood titer was 1:20,000.

The application of a serologic test to milk as a diagnostic technique for leptospirosis was described by van der Hoeden in 1955.[14] The clear whey obtained from a milk sample was diluted with a saline solution containing 0.4% formalin. Living leptospiral cultures were mixed with the whey dilutions and kept at room temperature until the following day. If examinations under a microscope with dark-field illumination revealed "typical clumping" of the spirochetes, the test was considered positive. The examination of blood and milk samples taken from 100 cattle infected with either *Leptospira grippotyphosa* or *Leptospira canicola* indicated the presence of a whey agglutinin titer approximately a tenfold dilution lower than the concomitant blood titer. Three cows with blood titers of 1:2,000, however, had negative whey titers. The whey titer was considered more specific since heterologous antigens always gave negative results. The whey test was recommended for use where the collection of blood would meet with technical difficulties or where a screening test was desired. Negative results with the whey test were not considered conclusive.

Morse *et al.*[8] reported on the correlation between the milk whey and blood serum titers of infected cattle. The milk whey was obtained through the action of rennet on skimmed milk samples held at 40 C. for 18 hours. Whey agglutinins were found at levels from 1:10 to 1:10,000 in a herd of cattle where the onset of leptospirosis had occurred about 2 months previously. The whey titers seemed to be higher in cattle which had recently commenced lactation. Whey reactions were not found in cattle with blood titers of 1:10 or 1:100. The higher whey titers found in recently infected herds and the persistence of the whey titers for "many months following infection" were emphasized.

Fennestad and Borg-Petersen[5] studied whey titers in heifers artificially infected with different leptospiral serotypes. The colostral whey titer was found to be 3 to 10 times higher than the serum titer, but after 24 hours the whey titer to serum titer ratio had dropped to 1:10 or 1:100. Whey agglutinins could not be detected at the 1:30 dilution in these heifers 26 days after parturition, in contrast to the finding of Morse *et al.*[8]

METHODS AND MATERIALS

Bulk tank herd milk samples from farms in the Ames area were examined in a preliminary survey through the courtesy of the Veterinary Hygiene Department, Iowa State University. No blood samples were obtained from these herds.

In the second phase of the work, blood samples were obtained from the local brucellosis testing laboratory and carefully identified as to name and address of herd owner, name and address of the veterinarian submitting the samples, and location by county and township. Only complete tests of dairy herds from the 33 counties within a 100-mile radius of Ames were selected. The samples were tested with the Stoenner plate-screen test[11] and herds having positive reactions were retested with the agglutination-lysis test.

Milk samples were obtained on the farm from the bulk storage tank or from the milk cans representing a single milking. Some quarter milk samples were also collected from individual cows. A herd history was procured from the herdsman or the veterinarian at the time of the visit.

Since the purpose of the investigation was to determine the feasibility of a screening test employing a composite herd sample, the herds were not selected in a random manner. An effort was made to include in the survey all categories from negative herds to herds with strong serologic indications and recent signs of leptospirosis infection.

The agglutination-lysis test was performed using the *pomona* strain of *L. pomona* as the antigen culture.* The antigen culture was maintained in Stuart's medium[12] as prepared by Difco Laboratories, Inc., and transferred every 4 to 6 days. Approximately 9 ml. of medium was placed in

* This culture was obtained from the WHO/FAO Leptospirosis Reference Laboratory, Division of Veterinary Medicine, Walter Reed Army Institute of Research, Washington, D.C.

sterile, screw-cap vials, 20 by 125 mm. in size. An inoculum of 1 ml. of an actively growing culture was added to each tube and the cultures were incubated for 4 to 6 days at 30 C. The antigen cultures were examined macroscopically for growth and, if suitable, were centrifuged at 500 g on a horizontal head for 10 minutes. The antigen was removed from the culture tube and examined for density with the dark-field microscope at a magnification of 150x. The antigen was diluted with buffered saline until it reached the optimum level of density as determined by microscopic observation. The optimum level was defined as a density which permitted the easy determination of a 50% end point. The *L. pomona* strain as cultivated usually required the addition of 4 to 6 ml. of buffered saline to 10 ml. of culture for adjustment to the correct density. The formula for the buffered saline was:

Na_2HPO_4	2.0 Gm.
KH_2PO_4	0.4 Gm.
NaCl	4.0 Gm.
H_2O (ion exchange)	1.0 liter

Serial dilutions were made in the buffered saline and 0.1 ml. of each dilution was placed in a small glass tube, 10 by 75 mm., and 0.1 ml. of antigen was added. Tenfold dilutions were prepared so as to produce preliminary dilutions of 1:5, 1:50, 1:500, 1:5,000, etc. which, after mixture with an equal volume of antigen, resulted in final dilutions of 1:10, 1:100, 1:1,000, 1:10,000, etc. The tubes were shaken and incubated for 2 hours at 37 C. The tests were read on placing a small oblong drop from each tube on a microscope slide and examined by dark-field microscopy at 150x magnification. The titer of the serum was determined by the highest dilution which had at least 50% agglutination.

The milk samples were collected in screw-capped vials and transported to the laboratory in a portable ice chest. After standing overnight at 4 C., the cream was removed by aspiration. Approximately 5 ml. of the skimmed milk was placed in a small test tube and 3 to 5 drops of a liquid rennet extract* was added. The tubes were agitated and placed in a water bath maintained at 37 C. for 2 to 4 hours. The clotted milk was gently loosened from the sides of the tube with a clean applicator stick and the whey was removed with a pipette after centrifugation. The whey samples were stored in the frozen state or tested immediately.

Agglutination-lysis tests on the whey were performed in the same manner as previously described except that a different dilution scheme was utilized. The lowest dilution examined was prepared by mixing equal amounts of whey and the antigen, producing a final whey dilution of 1:2. Subsequent twofold dilutions were prepared from the whey so as to accurately determine the agglutinin content of the whey. The whey dilutions were, therefore, 1:2, 1:4, 1:8 and so on to the end point titer. A different dilution scheme was used on the quarter milk samples of 1:10, 1:40, 1:160, etc.

The antigen used in the Stoenner test was prepared commercially.*

RESULTS

There were 2 stumbling blocks expected in the task of detecting leptospiral agglutinins in composite herd milk samples. First, the level of antibody in the milk of a serologically positive cow would be diluted out by the milk of several negative cows. The bulk tank storage method would exert an even greater dilution effect than the use of milk cans. It would be necessary for the test to detect antibody levels at very low dilutions. Second, the physical nature of the whey might interfere with the dark-field examination of the agglutination-lysis tests performed on it. This difficulty would be increased when lower dilutions of whey were examined. A preliminary study was performed on herd milk samples from dairy farms in the vicinity of Ames. It was reasonably expected that some of the herds would be infected with leptospirosis. A total of 30 milk samples was tested from herds where the bulk tank storage method was used. The whey titers obtained from these samples are presented (Table 1). A second set of samples was procured 4 months later for comparison with the results of the first test. The herds were identified by the initials of the owner.

The whey dilutions were prepared as described, commencing with a 1:2 dilution. The agglutination-lysis test on the 1:2 and 1:4 dilutions were occasionally difficult to read because of the light-scattering properties of substances in the whey. Otherwise, the tests were read in the usual manner. It helped to place thin drops on the slide and examine the edges of the drop when reading the tests under the dark-field microscope.

Three herds, W&M, CDT, and KH, were found to have fairly consistent titers at and above the 1:8 dilution on both tests. Negative titers were found on both tests for 18 herds. Herds DFA and FLM, negative on the first test, developed titers of 1:64 and 1:16, respectively, on the second test. Seven herds were classified as positive in the 1:2 or 1:4 dilutions on either or both of the 2 tests.

* Cheese Rennet, Chr. Hansen's Laboratory, Inc., Milwaukee, Wis.

* Serial Number 306124, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

The next logical step was to obtain a correlation between the blood serum titers and clinical signs of leptospirosis found in the individual cows with the whey titer detected in the herd milk sample (Table 2). This relationship is summarized from data obtained from 17 dairy herds. The herds are identified by a code number and listed in the order of ascending whey titers.

Herds 16 and 20 were considered negative controls for the investigation. However, herds 4 and 1 gave negative herd whey tests even though testing of some cattle in these herds revealed serum titers. Herd 1 was extremely large and the milk from the 8 cattle possessing titers of $1:10^3$ was diluted considerably by milk from the 117 negative cattle.

It was considered significant that herds 14 and 9, with a whey titer of 1:8, contained from the milk samples of herd 18 was of excellent quality, a Stoenner plate test was performed on it. All 3 milk can whey samples from this herd reacted at the 1:10 dilution of the Stoenner test.

Herd 15 was of interest in that it had a whey titer of 1:128 although only 2 cattle had titers of $1:10^3$ and $1:10^4$ and the remaining 13 cattle were negative. This herd was recently assembled, accounting for the absence of any indications of leptospirosis reported by the owner.

Following Stoenner's interpretation, herds 14, 3, 15, 17, 19, and 18 were considered "possibly infected" and had whey titers of 1:8 to 1:512. Two herds, 4 and 1, with titers of 1:40 and lower, failed to react to the whey test. Four herds with maximum Stoenner titers of 1:40 produced whey titers of 1:16 and 1:32.

TABLE 2—Relation of Composite Milk Sample Whey Titers to Serum Titers and Histories of 17 Dairy Herds

Herd No.	Agglutination-lysis test (No. of cattle per titer)							Stoenner plate test (No. of cattle per titer)					History*	Whey AL tests (titer)	
	N	10	10^2	10^3	10^4	10^5	10^6	N	10	40	160	640		Bulk tank	Milk** can
16	18	..	..	..	..	..	..	18	..	..	..	..	N	N	....
20	16	..	..	..	..	..	..	16	..	..	..	..	N	N	....
4	8	7	..	..	..	..	..	7	7	1	..	..	+V	....	N
1	117	2	16	8	..	..	..	122	12	9	..	..	N	N	....
14	9	3	7	3	2	..	..	18	3	1	2	..	++V	8	....
9	6	12	3	5	1	..	..	No test	..	..	..	..	++V	8	....
2	..	9	4	..	..	..	..	1	8	4	..	..	+V	16	....
11	5	1	7	5	2	..	..	9	6	5	..	..	+V	....	16
12	1	1	6	4	1	..	..	6	2	5	..	..	N	16	....
5	4	8	8	1	..	..	..	4	14	3	..	..	+V	....	32
7	7	4	3	2	..	..	..	No test	..	..	..	..	++V	....	32
8	2	6	5	4	3	..	..	No test	..	..	..	..	N	32	....
3	8	2	8	4	..	..	..	7	7	6	1	1	++V	....	4
15	13	..	..	1	1	..	..	13	1	..	..	1	N	....	128
17	15	5	3	9	..	..	..	20	..	4	3	5	++V	256	.....
19	....	..	1	3	6	2	2	....	2	4	4	4	++	256	.....
18	....	1	2	6	6	2	..	1	3	5	4	4	N	.....	512

* The herd history was coded as follows: N = no signs of leptospirosis, + = signs of leptospirosis at least one year previous to the collection of samples, ++ = current signs of leptospirosis, and V = vaccination with *L. pomona* bacterin. ** Highest milk can sample titer.

cattle with titers of $1:10^4$ and reported current signs of leptospirosis. Herd 2 contained 13 cattle all of which had titers of at least 1:10. The absence of cattle with negative reactions in this herd accounted for the 1:16 whey titer without current signs of leptospirosis.

The whey titers of 1:256 for the bulk samples of herds 17 and 19 and the highest milk can whey titer of 1:512 from herd 18 were examples of what might be expected from herds where active leptospirosis infection was present. Since the whey obtained

A limited investigation was made on the individual quarter titers developed by cattle which had blood agglutinin titers against *L. pomona*. The results are summarized (Table 3) with the cattle arranged in the order of ascending serum titers.

The most striking feature of the data was the variation in whey titer found for cows with the same serum titer. Cattle with a serum titer of 1:10 produced whey agglutinin titers of from negative to 1:40. Likewise, cattle with serum titers of 1:1,000 had whey titers of negative to 1:640. On the

other hand, the similarity of quarter titers found for the same cow was also noteworthy. An F value of

$$1.70\ (F_{.05} = 2.78, F_{.01} = 4.16)$$

was calculated for the variation among quarters on the same cow. Data on the stage of lactation of each cow were not obtained.

DISCUSSION

The ability of the whey agglutination-lysis test to detect leptospiral antibodies in bulk tank herd milk samples was amply demonstrated by the results (Table 1). Unfortunately, the serologic data and history of these herds were not available. Assuming a titer of 1:8 as indicating infection, 5 of the 30 herds may be classified as "possibly infected." An over-all infection rate of 4.9% for herds has been reported in Iowa.[3] This value compares with the infection rate of these herds.

As mentioned, some difficulty was occasionally experienced in reading the tests in the 1:2 and 1:4 dilutions of whey. The scattering of light from materials in the suspension made it difficult to see the leptospires. It was possible, however, to place a coverslip on the whey-antigen drop and examine under high power for leptospires which had not been agglutinated. The careful preservation of the samples through cooling and the use of an active rennet aided in the production of an almost water-clear whey sample.

The development of whey titers by herds DF and FLM (Table 1) on the second test, which were negative on the first test, would seem to indicate the onset of leptospirosis in these herds. The number of negative whey titers seems to indicate a resonable degree of specificity for the test.

The foremost question to be answered from the data (Table 2) was what should be the minimum titer used to indicate herds suspected of infection with leptospirosis. Two herds showing current signs of leptospirosis, agglutination-lysis titers of 1:40,[4] and Stoenner titers of 1:160, gave whey titers of 1:8. Considering the disease signs accompanied by recognized diagnostic titers in these herds, it would appear that this should be the critical dilution. From a practical standpoint, a dilution of 1:8 or 1:10 could be prepared directly instead of stepwise as in this study and a single agglutination-lysis test performed for each herd. Selection of a minimum titer of 1:16 or even 1:32 would designate as positive some herds in which leptospirosis was no longer in the active phase. However, a margin of error must always be allowed and a screening test should be designed so that so-called "false positive" rather than "false negative" readings are generally made.

TABLE 3—Agglutination-Lysis Test Results on Whey Quarter Titers and Blood Serum Titers of 19 Dairy Cows

Cow No.	Serum* titer	Quarter whey titer**			
		RF	RR	LR	LF
W22	10	N	N	N	N
0770	10	N	10	N	N
2878	10	N	N	10	10
9	10	10	10	10	10
0078	10	40	40	40	40
W62	100	N	N	N	N
W71	100	10	10	10	10
231010	100	10	10	10	10
5	100	10	40	10	10
1679	100	40	40	10	40
664206	100	40	40	40	40
8	100	40	160	160	160
4095	1,000	N	N	N	N
664204	1,000	10	10	10	10
W79	1,000	10	10	10	10
8047	1,000	40	40	40	40
4973	1,000	40	40	40	40
6545	1,000	160	160	160	40
8046	1,000	160	640	160	160

* Tenfold dilution scheme (1:10 through $1:10^6$). ** Fourfold dilution scheme (1:10 through 1:640).

It was impossible to evaluate the effect vaccination might have on the whey titers since all the vaccinations were performed on herds which reported present or past signs of leptospirosis. The absence of a positive history in herds 12, 8, and 18 must be considered in the light of the natural reluctance of some dairy herd owners to divulge information to representatives of government agencies. The owner of herd 12, however, kept swine in the dairy barn and lot. Abortions or other signs of leptospirosis were denied by the owner of herd 8, but he willingly supplied the information that his son's herd on an adjacent farm was infected. Herd 18 was pastured in a field with a common fence next to herd 19, which had severe signs of leptospirosis, and a small brook passed through the farm of herd 19 into the farm of herd 18.

The whey test on composite herd milk samples could be used to locate dairy herds infected with leptospirosis in the same manner as the brucellosis milk ring test. The technical difficulties of preparing whey from the milk samples and setting up agglu-

tination-lysis tests would be more than offset by the alternative problem of collecting and testing blood samples. The expense of finding an infected herd is calculated according to the number of clean herds which must be tested to locate it. Further experimental work should be done involving a larger number of herds than were included in this limited study. The low level of the leptospiral agglutinins in the composite herd milk sample would appear to discourage the use of macroscopic tests with killed antigen to detect them.

The whey titer of the individual cow seems to vary considerably in relation to the serum titer. It would have been more informative if the stage of lactation for each cow had been recorded. According to Morse *et al.*,[8] the agglutinins are highest in the first month of lactation and after the 4th month tend to drop off to 1:10 or negative. The absence of a significant difference between the quarter titers on the same cow was regarded as evidence that localized udder infection does not occur in leptospirosis. During the acute phase of the disease, when the leptospiremia occurs, there is a generalized antigenic stimulation of the animal body. The mammary gland shares in this effect equally in all 4 quarters. After the acute phase, antibody production begins and detectable levels may be found in the milk. Since there was no significant difference between the quarter titers, it would appear that a chronic infection of the udder, as is seen in brucellosis, does not occur. Infection of one quarter would tend to cause a higher antibody titer in the milk from it than from the other quarters.

The detection of dairy herds infected with leptospirosis would seem to be assisted by the efforts most dairy farmers make to keep their cows in different stages of lactation. An infected cow whose serum reacted to a titer of 1:160 on the Stoenner test could have its milk diluted by a number of cows producing negative milk and still be detected in the composite sample.

SUMMARY

Consideration was given to means by which antibodies against *Leptospira pomona* might be detected in the composite herd milk sample as obtained from the bulk tank or milk can. The agglutination-lysis test was used to successfully detect antibodies in the whey obtained from the herd sample. Blood samples were obtained from 17 herds and examined with the agglutination-lysis test and the Stoenner plate test. The herds were visited, histories obtained, and a composite herd milk sample collected.

From a comparison of the data obtained, it was concluded that a whey titer of 1:8 in in the composite herd sample was the critical titer to be used in screening for leptospirosis. Titers of 1:256 and 1:512 were detected in the milk of 2 herds in which current signs of leptospirosis infection were present.

Quarter milk samples were obtained from 19 cows having blood serum antibody titers. Statistical evaluation indicated that there was no significant difference between quarter whey titers of the same cow.

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SUMMARIO IN INTERLINGUA

Le Detection de Anticorpore Contra *Leptospira pomona* in Composite Specimens de Lacte Gregari

Esseva studiate le possibilitate de deteger anticorpore anti *Leptospira pomona* in le typo de composite specimen de lacte gregari que es obtenite ab le tank de collection o un cylindro de lacte. Le test de agglutination-lyse esseva usate a bon successo in le detection de anticorpore in lacte clar ab specimens gregari. Specimens de sanguine esseva obtenite ab 17 greges e examinate con le test de agglutination-lyse e le test a placa de Stoenner. Le greges esseva visitate, datos historic esseva obtenite, e composite specimens de lacte gregari esseva colligite.

Super le base de un comparation del datos obtenite il esseva possibile concluder que un titro de 1:8 in le lacte clar de un composite specimen gregari esseva le nivello critic a usar in le scrutinio pro le detection de leptospirosis. Titros de 1:256 e 1:512 esseva detegite in le lacte de duo greges in le quales signos de un currente infection per leptospira esseva presente.

Specimens de lacte de quartiero esseva obtenite ab 19 vaccas con titros de anticorpore in le sero del sanguine. Le evalutation statistic indicava que nulle significative differentia existeva inter le titros in le lacte clar del quartieros de un vacca particular.

Effect of Cortisone on *Leptospira pomona* Infection in the Guinea Pig

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LEPTOSPIRA POMONA possesses low pathogenicity for the guinea pig. It produces only a transient pyrexia and leptospiremia, but may induce leptospiruria of several months' duration.[18, 19] For this reason, most laboratories use guinea pigs or other susceptible animals to demonstrate leptospires microscopically and culturally in either peritoneal fluid or heart blood during the first few days after infection, rather than attempt to produce overt signs of disease in the animal.[6]

Cortisone given in large doses may depress the resistance of animals to a wide variety of infections.[4, 8, 9, 16, 17] Cortisone may also activate latent infections or render animals susceptible to fatal infection by agents which are normally nonpathogenic or nearly so.[5, 11] The decreased resistance has also facilitated the isolation of some infectious agents in laboratory animals.[3, 10] The exact mechanism by which cortisone depresses resistance to infection is not known, but some authors[4, 8] believe that suppression of inflammation accounts for much of the effect. Some of the inflammatory responses suppressed by cortisone administration are: capillary permeability, cellular infiltration, fluid exudation, fibrinogenesis, granulation, and repair.

The purpose of this investigation was to determine the effects of varying dosage levels and sequences of administration of cortisone in young guinea pigs infected with *L. pomona*.

MATERIALS AND METHODS

Guinea pigs approximately 1 month old and weighing 200 to 270 Gm., were used in this experiment. They were all obtained from the same commercial source. They were fed commercial guinea pig pellets.*

The *L. pomona* (Univ. of Ill. Vet. Diagnostic Lab. Acc. No. T79013) used to infect the guinea pigs was originally isolated from aborted porcine fetuses from a central Illinois farm in 1957. The inoculum consisted of a pure broth culture of *L. pomona* which had been incubated at 29 C. for 7 days in Stuart's medium.

In the first experiment, the guinea pigs were allotted to 6 groups of 6 animals each (groups 1, 2, 3, 4, 5, and 6), 1 group of 10 animals (group 7), and 3 groups of 5 animals each (groups 8, 9, and 10). The guinea pigs in group 1 were given 10 mg. of cortisone* per day beginning 3 days before inoculation with *L. pomona*. Each animal in group 2 was given 20 mg. of cortisone daily beginning 3 days before exposure to *L. pomona*. Guinea pigs in groups 3 and 4 were given daily doses of 10 mg. and 20 mg., respectively, beginning the day of inoculation. Guinea pigs in groups 5 and 6 were given 10 mg. and 20 mg. of cortisone, respectively, beginning 3 days after inoculation of leptospires. All cortisone injections were given subcutaneously each day until 21 days after inoculation. At this time, all surviving animals were killed. Guinea pigs in group 7 were the controls infected with *L. pomona*. Those in groups 8 and 9 were uninfected and were given 10 mg. and 20 mg. of cortisone, respectively, for 21 days. Guinea pigs in group 10 were the untreated and uninfected controls. All guinea pigs in groups 1 to 7 were exposed to *L. pomona* at the same time, each being given 0.5 ml. of *L. pomona* inoculum intraperitoneally.

Rectal temperatures were recorded at approximately the same time each day. The guinea pigs were weighed at 6-day intervals. Necropsies were performed and gross lesions recorded for those that died during the experiment and those killed at the end of the experiment.

Blood samples were obtained from the surviving guinea pigs in each group by cardiac puncture at 4, 8, 14, and 21 days after inoculation. Approximately 1.5 ml. of blood was collected aseptically from each animal. One or 2 drops of blood was cultured in each of 2 tubes containing 2 ml. of Stuart's medium with 10% inactivated (56 C. for ½ hour) and filtered horse serum. The remaining serum from each sample was frozen and later used to conduct the agglutination-lysis test modified from the procedure of Schuffner and Mochtar.[14] Serum was diluted in 0.15 M NaCl solution to make final dilutions of 1/10, 1/100, 1/1,000, 1/10,000, and 1/100,000. A 7-day-old live culture of *L. pomona* (strain P54874) was used as antigen.[1] This strain had been isolated from aborted porcine fetuses

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* Guinea Pig Chow, Ralston-Purina Co., St. Louis, Mo.

* Cortone acetate, U.S.P., Merck Sharp & Dohme, Philadelphia, Pa.

from a central Illinois farm in 1953 and had been maintained in Stuart's medium. To tubes containing 0.1 ml. of each dilution of serum (5 per serum sample), 0.1 ml. of antigen was added. The serum-antigen mixtures were incubated at room temperature for approximately 4 hours. A serum reaction was regarded as positive when agglutination or lysis occurred in 50% or more of the organisms observed by darkfield microscopy at a magnification of 120 x.

The liver, spleen, and kidneys were ground and suspended in sterile NaCl solution and 1 or 2 drops of this suspension was cultured in each of 2 tubes of Stuart's medium (liver and spleen together). Urine was also cultured in Stuart's medium. The blood and tissue cultures in Stuart's medium were incubated at 29 C. and examined for the presence of leptospires of darkfield microscopy (magnification 120 x) at weekly intervals for 4 weeks, and again at 6 weeks and 8 weeks. Cultures were considered negative when no organisms could be detected during the 8 weeks.

Samples of heart blood of animals dead less than 12 hours were cultured on blood agar (Difco) plates.

In experiment 2, the 15 guinea pigs in group 1 were each given 20 mg. of cortisone per day for 21 days beginning on the day *L. pomona* was inoculated. The 15 guinea pigs in group 2 were inoculated with *L. pomona* culture. In group 3, there were 4 guinea pigs given 20 mg. of cortisone for 21 days. From animals dead less than 8 hours, clotted heart blood, rather than liver and spleen suspensions, was cultured for leptospires. Heart blood of all live guinea pigs was cultured on blood agar plates (Difco) at 8, 14, and 21 days postinoculation and a fecal sample from each was cultured in selenite broth (Difco). Heart blood and feces of animals that died were also cultured. All organisms isolated from these mediums were subjected to biochemic and serologic tests. Biochemic tests were for urea, glucose, lactose, maltose, sucrose, dulcitol, inositol, and trehalose broths and Kligler's iron agar (Difco). All cultures were incubated at 37 C. for 24 hours. Organisms producing biochemic reactions characteristic of Salmonella were typed.* *Salmonella enteritidis* was the only member of the genus found.

RESULTS

Experiment 1.—Body temperatures of all animals given leptospires in groups 1, 2, 3, 4, 5, 6, and 7 were moderately elevated. Peaks of 104.5 to 105.5 F. were reached 2 to 6 days after inoculation. By the 8th day, temperatures of all inoculated animals had returned to normal. Group mean daily temperatures remained in the range of 102.0 to 103.4 F. throughout the remainder of the experiment. Temperatures of leptospiremic animals at 14 and 21 days after inoculation were no higher than the temperatures of the animals which had developed localized renal infections.

Each animal was weighed 4 days before the beginning of the experiment and at subsequent 6-day intervals. Untreated guinea pigs (group 10) gained the most weight during the experiment, while those given only 10 mg. of cortisone per day (group 8) gained almost as much as the untreated animals. The guinea pigs in the inoculated control group and those in inoculated groups given cortisone had lower weight gains. Of special interest was the effect on guinea pigs given 20-mg. daily doses of cortisone. These animals usually had the lowest weight gains. The mean weight increase is summarized (Table 1).

Guinea pigs given cortisone in addition to leptospires remained in the leptospiremic phase of the disease longer than those not given cortisone. Groups 3 and 4 containing guinea pigs given cortisone and leptospiral culture on the same day, had the greatest number of leptospiremic guinea pigs 8 days after treatment. However, the groups of guinea pigs given the higher dosage (2, 4, and 6) of cortisone had more guinea pigs with leptospiremia 21 days after inoculation. Table 2 shows the number of infected animals with leptospiremia in each group at 4, 8, 14, and 21 days following exposure.

Although leptospiremia was prolonged in guinea pigs given cortisone, the hormone did not reduce resistance to the extent that *L. pomona* (Univ. of Ill. T79013) killed guinea pigs with regularity. Only 2 dead

TABLE 1—Cortisone Treatment Schedules of both L. pomona-Inoculated and Uninoculated Guinea Pigs and Mean Group Body Weight Increases Occurring During the 24-Day Observation Period in Experiment 1

Group	Cortisone treatment		*L. pomona* inoculation	Mean body wt. increase (Gm.)
	mg./day	daily from		
1	10	Day 1 to 24	+*	107
2	20	Day 1 to 24	+	84
3	10	Day 3 to 24	+	120
4	20	Day 3 to 24	+	98
5	10	Day 6 to 24	+	75
6	20	Day 6 to 24	+	93
7	....	.....	+	118
8	10	Day 3 to 24	—	160
9	20	Day 3 to 24	—	107
10	....	.....	—	164

* Each guinea pig was given intraperitoneally 0.5 ml. of an 8-day-old Stuart's medium culture of *L. pomona* on day 3 of the experiment.

* Salmonella typed by Department of Health, Education, and Welfare, Public Health Service Communicable Disease Center Laboratory, Chamblee, Ga.

TABLE 2—Cortisone Treatment Schedules and Number of Guinea Pigs in Experiment 1. Leptospires in Circulating Blood Determined by Blood Cultures at 4, 8, 14, and 21 Days after *L. pomona* Inoculation

Group	Cortisone treatment		Blood cultures after *L. pomona* inoculations*			
	mg./day	daily from	4th day	8th day	14th day	21st day
1	10	Day 1 to 24	6/6**	4/6	2/4	3/4
2	20	Day 1 to 24	6/6	3/5	2/5	3/3
3	10	Day 3 to 24	6/6	6/6	1/5	0/4
4	20	Day 3 to 24	6/6	5/5	3/4	3/4
5	10	Day 6 to 24	6/6	2/6	0/6	1/6
6	20	Day 6 to 24	6/6	2/6	2/6	3/4
7	....	.....	10/10	4/8†	1/8	0/8

* *L. pomona* culture was given to guinea pigs on day 3 of the experiment. Thus, the 4th day after *L. pomona* inoculation was day 7 of the experiment, the 8th day after *L. pomona* inoculation was day 11 of the experiment, the 14th day after *L. pomona* inoculation was day 17 of the experiment, and the 21st day after *L. pomona* inoculation was day 24 of the experiment.

** Numerator is number of blood cultures positive for leptospires. Denominator is number of animals in the group.

† Two guinea pigs died as a result of cardiac puncture.

guinea pigs (1 in group 2, 1 in group 4) had signs and lesions typical of fatal leptospirosis: icterus and hemorrhages in the lungs and heart, kidneys, and liver. Guinea pigs dead after the 8th day did not have lesions of leptospirosis. *Escherichia coli* and a Streptococcus producing *beta* hemolysis were cultured from guinea pigs having lesions of pneumonia. These died later in the experiment. Unfortunately, the tissues of some dead guinea pigs were not cultured until 8 hours after death. In all animals that died, cultures of liver and spleen were negative for leptospires, even though in a few cases blood cultures were positive within the same 24-hour period. The fact that some tissues were not cultured for several hours after death may account for the low recoverability of leptospires. Recovery of leptospires from either kidney tissue or urine was considered evidence of renal infection. There were no deaths in the control groups of guinea pigs given only cortisone (8 and 9), or the untreated control group (10). At necropsy, none of the guinea pigs of groups 8, 9, and 10 had lesions of leptospirosis.

Leptospiremia existed 21 days after inoculation in the presence of agglutination-lysis titers as high as 10^5. Among groups of infected guinea pigs given cortisone as well as those not given cortisone, the serum titer sometimes decreased between 8 and 14 days after inoculation and then increased by the 21st day. Recovery of leptospires from the blood of infected guinea pigs at 4, 8, 14, and 21 days and the agglutination-lysis titers at 8, 14, and 21 days after inoculation are summarized (Table 3).

Experiment 2.—The initial febrile response of the guinea pigs was similar to that in the first experiment. The maximum daily mean group temperatures during the first 8 days after inoculation were 103.7 F. for the cortisone-infected group, 104.7 F. for the infected control group, and 102.5 F. for the cortisone control group. Guinea pigs in both infected groups reached their mean maximum temperatures between 2 and 3 days after exposure. The mean daily temperatures of animals in the infected control group remained between 101.8 and 102.9 F. during the remainder of the experiment. The mean daily temperatures of the cortisone control guinea pigs during the course of the entire experiment ranged from 101.8 to 102.9 F. Following the 10th day after exposure, the temperatures of the cortisone-infected guinea pigs began to rise. From the 10th to the 21st day, the mean daily temperature readings varied from 103.1 to 104.8 F. with the peak occurring on the day 15.

The mean group body weights were similar to those of animals in experiment 1. The infected guinea pigs given cortisone gained the least.

In experiment 2, leptospires were not recovered 14 days after inoculation from the blood of guinea pigs given cortisone, but the cortisone-treated animals remained leptospiremic longer than the untreated-infected controls. Eight days after exposure, 6 of 9 animals in the cortisone-infected group had leptospiremia compared with only 2 of the 15 infected control animals. The number of guinea pigs in each group with leptospiremia at 4, 8, 14, and 21 days

after *L. pomona* inoculation are shown (Table 4).

Heart blood of all dead animals was cultured on blood agar. In all cases of death of guinea pigs 12 days after exposure, *S. enteritidis* was cultured. Many leptospiral cultures were heavily overgrown with bacterial contaminants, possibly accounting for the lower rate of isolation of leptospires from these animals.

At termination of the experiment, 21 days after inoculation, the heart blood of all surviving animals was sterile. The intestinal contents of the infected or cortisone-control guinea pigs were cultured and found to contain *S. enteritidis*. The sur-

TABLE 3—Guinea Pig Blood Cultures and Agglutination-Lysis Serum Titers at 4, 8, 14, and 21 Days After L. pomona Inoculation in Experiment 1

Group No.*	Animal No.	4 days	8 days		14 days		21 days	
		Blood	Blood	Titer	Blood	Titer	Blood	Titer
	1	+	+	10^{3}**	+	10^{3}	+	10^{5}
	2	+	+	10^{3}	+	10^{3}	+	10^{4}
1	3	+	−	10^{3}	−	10^{3}	+	10^{4}
	4	+	+	10^{3}	†	....	....	....
	5	+	+	10^{2}	†	....	....	....
	6	+	−	10^{3}	−	10^{3}	−	10^{3}
	7	+	+	10^{3}	−	10^{3}	†	....
	8	+	+	10^{3}	−	10^{5}	+	10^{5}
2	9	+	−	10^{5}	+	10^{3}	+	10^{4}
	10	+	+	10^{3}	−	10^{3}	+	10^{3}
	11	+	−	10^{4}	+	10^{3}	†	....
	12	+	†	....	....	....	....	....
	13	+	+	10^{3}	+	10^{4}	−	10^{4}
	14	+	+	10^{3}	−	10^{3}	−	10^{4}
3	15	+	+	0	−	10^{3}	†	....
	16	+	+	0	−	10^{3}	−	10^{4}
	17	+	+	10^{3}	−	10^{3}	−	10^{3}
	18	+	+	10^{3}	†	....	....	....
	19	+	+	10^{4}	+	10^{3}	−	10^{4}
	20	+	†	....	....	....	....	....
4	21	+	+	10^{4}	+	10^{3}	+	10^{3}
	22	+	+	10^{3}	−	10^{3}	+	10^{3}
	23	+	+	0	†	....	....	....
	24	+	+	0	+	10^{3}	+	10^{3}
	25	+	−	10^{3}	−	10^{3}	−	10^{4}
	26	+	−	10^{4}	−	10^{3}	+	10^{4}
5	27	+	−	10^{3}	−	10^{3}	−	10^{3}
	28	+	+	10^{5}	−	10^{4}	−	10^{4}
	29	+	−	10^{3}	−	10^{3}	−	10^{4}
	30	+	+	10^{3}	−	10^{3}	−	10^{4}
	31	+	−	10^{3}	−	10^{3}	−	10^{5}
	32	+	−	10^{3}	−	10^{3}	†	....
6	33	+	−	10^{4}	−	10^{3}	+	10^{5}
	34	+	+	10^{3}	+	10^{3}	†	....
	35	+	+	10^{3}	+	10^{3}	+	10^{5}
	36	+	−	10^{3}	−	10^{3}	+	10^{3}
	37	+	−	10^{4}	−	10^{3}	−	10^{4}
	38	+	+	10^{5}	−	10^{5}	−	10^{5}
	39	+	†	....	....	....	....	....
	40	+	+	10^{3}	+	10^{3}	−	10^{4}
7	41	+	+	10^{3}	−	10^{4}	−	10^{4}
	42	+	−	10^{5}	−	10^{3}	−	10^{5}
	43	+	+	10^{5}	−	10^{5}	−	10^{4}
	44	+	−	10^{5}	−	10^{5}	−	10^{4}
	45	+	†	....	....	....	....	....
	46	+	−	10^{4}	−	10^{3}	−	10^{3}

* Group 1—10 mg. of cortisone per day starting 3 days before *L. pomona* inoculation and for 24 days.
Group 2—20 mg. of cortisone per day starting 3 days before *L. pomona* inoculation and for 24 days.
Group 3—10 mg. of cortisone per day starting day of *L. pomona* inoculation and for 21 days.
Group 4—20 mg. of cortisone per day starting day of *L. pomona* inoculation and for 21 days.
Group 5—10 mg. of cortisone per day starting 3 days after *L. pomona* inoculation and for 18 days.
Group 6—20 mg. of cortisone per day starting 3 days after *L. pomona* inoculation and for 18 days.
Group 7—Leptospire culture only.

** Titers expressed as reciprocal of highest serum dilution producing 50% or more of agglutination and lysis or both.

† Died before this day. + = Culture positive for leptospires; − = culture negative for leptospires.

viving guinea pig in the cortisone-infected group also had *S. enteritidis* in its intestinal contents.

The death rate was higher in the cortisone-Leptospira inoculated group in experiment 2 than in experiment 1. There were no deaths in either the infected or cortisone control guinea pigs.

TABLE 4—Number of Guinea Pigs with Leptospiremia at 4, 8, 14, and 21 Days After L. pomona Inoculation in Experiment 2

Group*	4 days	8 days	14 days	21 days
1	15/15**	6/9	0/4	0/1
2	15/15	2/15	0/15	0/15

* Group 1—20 mg. of cortisone per day starting day of *L. pomona* inoculation and for 21 days. Group 2—leptospire culture only.

** Numerator is number of blood cultures positive for leptospires; denominator is number of guinea pigs in group.

DISCUSSION AND CONCLUSIONS

The course of infection of *L. pomona* in guinea pigs as shown by the infected control guinea pigs, group 7 in experiment 1, and group 2 in experiment 2, was similar to the work reported earlier.[18,19] A febrile reaction developed the day following inoculation and was present for about 7 days. During the febrile period, leptospires could be recovered from the circulating blood; shortly thereafter, the organisms could be isolated only from the urine and kidneys. There were no deaths due to leptospirosis and no gross lesions of the infection among guinea pigs killed 21 days after exposure.

In experiment 1, all cortisone-infected guinea pigs developed a febrile response similar to the infected control animals. The guinea pigs which had leptospiremia at 14 and 21 days after exposure did not have elevated temperatures. This finding suggested that cortisone may impair the febrile response. The same observation has been made in connection with other infectious diseases.[2,7]

The febrile responses of guinea pigs in experiments 1 and 2 were similar. However, during the activation of the Salmonella infection in experiment 2, the body temperatures reached peaks of over 105.0 F. In this study, cortisone appeared to have little or no effect on the febrile reaction to primary acute infections.

In experiment 1, the guinea pigs that were infected and were given 20-mg. doses of cortisone (2, 4, and 6), gained the least weight; however, guinea pigs of group 5, given 10-mg. doses for the shortest period (18 days), had the lowest group mean weight increase. Although all guinea pigs in group 5 lived, the effects of leptospirosis and cortisone treatment were reflected in the depression of average gains. In experiment 1, the surviving animals in groups 1 and 3 gained as much as the untreated-infected controls.

In experiment 1, the groups of guinea pigs given the 20-mg. daily dosage of cortisone (groups 2, 4, and 6) had the greater number with leptospiremia at 21 days after inoculation. Apparently, the 20-mg. dosage interfered with the ability of the animals to resist the infecting agent. Guinea pigs of group 1, given the 10-mg. daily dosage for 24 days, also had a high proportion of leptospiremic animals at 21 days following exposure. The longer cortisone was administered, the less the animal was able to resist the infective agent.[9]

The apparent reactivation of leptospiremia in some animals occurred in the presence of agglutination-lysis titers as high as 10^5. The finding suggests that leptospiremia was not terminated completely by the level of the specific antibody in the circulating blood, and also confirms the findings of Morse *et al.*[12]

Antibody titers of some infected animals, irrespective of cortisone treatment, decreased between the 8th and 14th day after inoculation and increased again by the 21st day (Table 3). Morse *et al.*[12] theorized that *in vivo* lysis of large numbers of leptospires causes neutralization of circulating antibodies.

Antibody production has been claimed by some to be inhibited or reduced during cortisone administration,[15] while others believe there is no significant interference with antibody production even though total plasma protein decreases.[13] The results of this study suggested that even large dosages of cortisone, when started before or after infection, do not significantly influence specific antibody formation.

High dosages (20 mg.) of cortisone did not significantly affect the mortality rate of guinea pigs infected with *L. pomona* (Univ. of Ill. T79013), although 1 guinea pig dead at 7 days and 1 dead at 8 days after inoculation had lesions of fatal leptospirosis. Cortisone prolonged the period

of leptospiremia, but did not influence the eventual outcome.

The activation of a latent intestinal pathogen by cortisone has been mentioned. However, in experiment 2, cortisone in combination with *L. pomona* (Univ. of Ill. T79013) appeared to activate *S. enteritidis*, producing a fatal septicemia in 14 of 15 guinea pigs.

SUMMARY

It was found that cortisone in 10- and 20-mg. doses given for 18, 21, or 24 consecutive days, lowered the resistance of young guinea pigs to *Leptospira pomona* infection. The morbidity rate was greater in the cortisone-treated guinea pigs as reflected in the lower weight gains, but death accompanied by icterus and fever, and by renal, hepatic, and vascular failure was not produced consistently. However, the resistance of animals was lowered sufficiently by cortisone to permit the leptospires to remain in the circulating blood 2 weeks longer than in untreated guinea pigs infected with leptospires.

The prolonged use of cortisone apparently enabled the leptospires to re-enter the circulating blood 1 to 2 weeks after Leptospira-negative blood samples had been obtained. The recurring leptospiremia took place in spite of agglutination-lysis serum titers as high as 10^5. Cortisone did not significantly interfere with the formation of agglutination-lysis serum titers.

In a 2nd experiment, cortisone and *L. pomona* activated a latent *Salmonella enteritidis* infection, causing septicemia and a high mortality rate. Neither cortisone nor the leptospiral infection alone activated the Salmonella infection.

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SUMMARIO IN INTERLINGUA

Effecto de Cortisona Super Infection per *Leptospira pomona* in le Porco de India

Esseva constatate que cortisona in doses de 10 e 20 mg per die in 18, 21, o 24 consecutive dies reduceva le resistentia de juvene porcos de India contra infectiones per *Leptospira pomona*. Le morbiditate esseva plus general in le porcos de India tractate con cortisona, a judicar per lor reducite ganios in peso. Sed morte accompaniate de ictero e febre e de disfallimento renal,

hepatic, e vascular non esseva producite uniformemente. Tamen, le resistentia del animales esseva reducite sufficientemente per le cortisona pro permitter que le leptospiras remaneva in le sanguine circulante 2 septimanas plus longe que in le non-tractate porcos de India inficite con leptospiras.

Le prolongate uso de cortisona apparentemente permitteva al leptospiras re-entrar in le sanguine circulante inter 1 e 2 septimanas post que specimens de sanguine negative pro leptospira habeva essite obtenite. Le recurrente leptospiremia occurreva in despecto de titros de lyse de agglutination attingente maximos de usque a 10^6. Cortisona non interfereva significativemente in le formation del titros seral de lyse de agglutination.

In un secunde experimento, cortisona e *L. pomona* activava un latente infection per *Salmonella enteritidis*, causante septicemia e un alte mortalitate. Ni cortisona ni le infection leptospiral per se activava le infection per salmonella.

Estimation of Low Concentrations of Plasma Glucose Using Glucose Oxidase

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GLUCOSE OXIDASE specifically oxidizes glucose and, in conjunction with peroxidase, will transfer oxygen to suitable chromogenic receptors. The principle has been applied to the determination of blood glucose concentrations in human clinical studies.[3,4,6] These methods have procedural disadvantages,[7] and lack ability at concentrations below 50 mg./100 ml.[4] These disadvantages have been reduced in the present method with considerable benefit at low concentrations. The method is now suitable for studies on ruminant animals, and has been used to reevaluate changes in blood sugar which occur during bovine ketosis and ovine pregnancy toxemia.

MATERIALS AND METHODS

Blood samples were taken by venipuncture into oxalate-fluoride anticoagulant. The blood was centrifuged, and the plasma stored in a deep freeze. After thawing, protein-free filtrates were made using either $Ba(OH)_2$–$ZnSO_4$ or $ZnSO_4$–NaOH. A series of test tubes were set in a rack; 0.4 ml. of each unknown filtrate was added to each of 2 test tubes; then 4.0 ml. of the enzyme-dye reagent was added. Blanks consisted of 0.4 ml. of water plus reagent and standards of 0.4 ml. of a 40 μg./ml. glucose solution plus reagent. The tubes were shaken and placed for 30 minutes in a water bath at 37 C. After cooling, the orange color was read at 420 mμ in a Beckman "B" spectrophotometer.

The enzyme-dye reagent was made with 125 mg. of glucose oxidase,* 5 mg. of peroxidase,** and 0.5 ml. of a 1.0% solution of 3,3′-dimethoxybenzidine in 95% ethanol, the volume being made up to 100 ml. with 0.4 M pH 7.0 phosphate buffer. The solution was mixed, filtered, then kept in a dark glass bottle in the refrigerator. The reagent darkened upon storing and was usable for only a week after preparation. In order to minimize expense, the cruder Sigma glucose oxidase was used. When tested manometrically, the theoretical uptake of oxygen to convert glucose to gluconic acid was complete in 20 minutes at 37 C.

Glucose was also estimated by the Nelson-Somogyi method.[5]

RESULTS

A series of 7 filtrates were made from a single sample of bovine plasma, enough glucose was then added to the plasma sample to increase its concentration by 20 mg./100 ml. and another 7 filtrates made. The mean ± s.d. of each group was 54.3 ± 0.173 and 73.8 ± 0.214 mg./100 ml., respectively. The recovery of glucose was thus 97.7 ± 0.5%. The standard error for glucose estimations done in duplicate would be 0.14 mg./100 ml.

The ability of the enzyme method to estimate glucose at low concentrations was tested with a series of glucose standards (Fig. 1). Beer's law was obeyed almost perfectly by the oxidase estimations, while the Nelson-Somogyi method underestimated glucose at low concentrations.

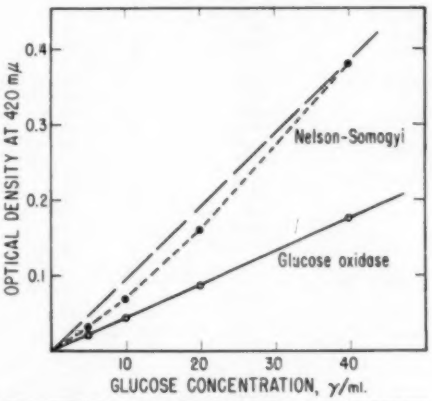

Fig. 1—Optical densities corresponding to a series of glucose concentrations determined by the glucose oxidase and Nelson-Somogyi methods; a solution of 40 μg./ml. corresponds to a plasma concentration of 40 mg./100 ml. Note that Beer's Law plots a straight line, so that the Nelson-Somogyi method underestimated glucose at low concentrations.

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Plasma glucose concentrations were estimated by the 2 different methods on a series of samples from cows and sheep (Table 1). In only 3 cases was the estimate by the Nelson-Somogyi method less than the corresponding estimate by the glucose oxidase method. Two of these cases were at low concentrations where the Nelson-Somogyi method probably gave an underestimate of the true concentration (Fig. 1). The other case at a higher concentration cannot be accounted for except by experimental error.

The difference between estimates by the 2 methods on the same sample might be due to other similar sugars sometimes called "saccharoids," such as galactose or fructose. These differences were between 0 and 12 mg./100 ml. for 90% of the estimates; the mean was 6 mg./100 ml. The differences for lactating cows ranged slightly higher and those for ketotic cows slightly lower than those for nonlactating cows, pregnant sheep, and sheep with pregnancy toxemia; this discrepancy becomes insignificant if the differences are expressed as a percentage of the Nelson-Somogyi estimates.

TABLE 1—Plasma Glucose Concentration Estimated by 2 Different Methods

Animals	Glucose oxidase glucose, mg./100 ml.	Nelson-Somogyi glucose, mg./100 ml.
Nonlactating cows	52.5	63
	44.2	48
	38.8	51
	50.5	51
	46.3	47
Lactating cows	44.2	52
	46.3	63
	48.3	56
	48.3	59
	50.5	56
Ketotic cows	33.6	39
	25.2	29
	23.2	28
	10.5	7
	38.2	38
Pregnant sheep	73.8	76
	40.0	50
	56.2	62
	53.8	64
	74.0	70
	40.4	42
	39.4	46
	35.3	42
Toxemic sheep	24.2	25
	91.3	101
	20.2	28
	33.7	38
	15.2	16
	81.6	97
	300+	300+
	16.8	12

DISCUSSION

Other methods have often been used in the past to demonstrate low concentrations of blood sugar in bovine ketosis and ovine pregnancy toxemia, and occasional high concentrations in the latter disease. These methods have not discriminated between glucose, galactose, and fructose. The possibility has existed that changes in blood sugar concentration may not have been due to glucose.

Lactose has been found in the blood of lactating cows,[2] and if it was hydrolyzed or if for any other reason galactose was present in the blood,[2] it could induce hypoglycemia, as it appears to do in human congenital galactosemia.[1] The saccharoid level found in lactating and ketotic cows was not greater than that of nonlactating cows, so the hypoglycemia of bovine ketosis is unlikely to be due to galactose.

Fructose is present in high concentrations in ruminant fetal fluids. It has been possible that in advanced cases of ovine pregnancy toxemia, the placental barrier might break down, with fructose passing into the maternal blood, and contributing to the extraordinarily high concentrations of sugar which are sometimes found. Again, the saccharoid levels found in pregnancy toxemia render this possibility unlikely.

SUMMARY

A method for estimating plasma glucose concentrations specifically with glucose oxidase has been developed to give a standard error for duplicates of less than 0.2 mg./100 ml. and to follow Beer's law down to as low as 5 mg./100 ml. Comparison with estimates by the Nelson-Somogyi method showed that glycemic changes during bovine ketosis and ovine pregnancy toxemia are due mainly to glucose, not galactose or fructose.

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SUMMARIO IN INTERLINGUA

Le Estimation de Basse Concentrationes de Glucosa del Plasma, con le Uso de Oxydase Glucosic

Esseva disveloppate un methodo pro le estimation de concentrationes plasmatic de glucosa, specificamente con le uso de oxydase de glucosa, resultante in un error standard pro repetitos de minus que 0,2 mg per 100 ml e sequente le lege de Beer in basso usque a 5 mg per 100 ml. Le comparation con estimationes effectuate secundo le methodo de Nelson-Somogyi monstrava que alterationes glycemic in cetosis bovin e in toxemia de pregnantia ovin es causate primarimente per glucosa, non per galactosa o fructosa.

Erythrocyte Survival in Experimental Anaplasmosis

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THE CLINICAL manifestations of *Anaplasma marginale* infection in cattle are generally well known, but the mechanism inducing the central change, anemia, has not been adequately elucidated. Foote [8] concluded that there is no evidence of hemolysis and suggested that the anemia was due to depression of hemopoiesis. This hypothesis did not receive wide acceptance since it disregarded the pathologic findings in the liver and spleen which indicate red blood cell (r.b.c.) destruction.[6] In a study utilizing Chromium-51 (Cr^{51})-labeled red blood cells, Hansard and Foote [9] later concluded that the anemia was due to depression of hemopoiesis or increased removal of r.b.c. Brock [4] has presented data indicating that a slight decrease in hemopoiesis may occur early in the course of infection, although his investigation showing increased fecal urobilinogen excretion supports the theory that increased hemolysis plays the major role.

The present report presents data obtained with Cr^{51}-labeled erythrocytes which indicate that increased hemolysis alone can account for the anemic manifestations in anaplasmosis.

MATERIALS AND METHODS

Chromium-51 was obtained as a sterile isotonic sodium chromate solution.* Red blood cells to be labeled were drawn into acid citrate-dextrose solution.* An appropriate amount of radioactive sodium chromate was added and the mixture incubated at room temperature with constant stirring for 1 hour. Fifty to 200 ml. of blood (15 to 60 ml. of r.b.c.), depending on the size and hemoglobin level of the animals, were labeled with 200 to 600 μc. of Cr^{51}. The specific activity was such that it was not necessary to exceed 10 to 20 μg. of chromium per milliliter of r.b.c. After incubation, 50 to 150 mg. of ascorbic acid in isotonic solution was added to stop the labeling process. In all but one case, this procedure resulted in 70 to 90 per cent incorporation of the isotope in r.b.c. The labeled blood was then transfused into the appropriate animal via the jugular vein.

Samples were collected from the animals in heparinized syringes. Whole blood and plasma were counted in 2-ml. amounts and corrections then made for Cr^{51} within the r.b.c. by use of the packed cell volume of the same sample. All counting was done in a well scintillation counter* attached to a Tracerlab 1000 scaler.

To determine blood volumes, 2 to 4 samples were taken during the first hour after transfusion of the labeled blood. The activity of the 24-hour sample was then taken as 100 per cent for plotting of the Cr^{51} clearance rate.

The blood cell counts, packed cell volumes (PCV), hemoglobin levels, and Anaplasma body counts were conducted by standard methods. Complement-fixation tests were performed with antigen supplied by the Agricultural Research Service, U.S. Department of Agriculture.

Five normal Hereford cattle, 8 to 15 months old, were used to establish normal values. To compare the clearance rates of intact cattle with splenectomized subjects, 3 animals, 8 to 12 months old, that had been splenectomized several weeks previously were used. Six splenectomized animals, 8 to 12 months old, were studied during various phases of the anaplasmosis process. Two of these animals had been studied previously as splenectomized control animals.

RESULTS

Intact Control Animals.—Data for the 5 animals in this group are presented (table 1). For purposes of clarity, figure 1 depicts only 1 animal. The clearance curve consisted of 2 components during the first few days. This could be separated by subtraction into an early rapid component of short duration (R1) and a slower continuous component (R2). The cause of the rapid component is not known with certainty but may be due to an excess of Cr^{51} in the cells, resulting in 2 elution rates during this period.[10] During the first 24 hours following transfusion, 15 to 20 per cent of the injected Cr^{51} was excreted. While not confirmed in cattle, it has been shown that 95 to 100 per cent of the injected isotope can be accounted for in sheep at any time by adding the

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* Supplied by Abbott Laboratories, North Chicago, Ill.

* H. O. Angers, University of California, Berkeley.

amount remaining in the circulation to the sum of that excreted in urine and feces.[2] In view of the above, the turnover rate of Cr^{51}-labeled r.b.c. is considered to be a relative rate and not an absolute value. The mean half-time value of the continuous component was 11.7 days with a range of 10.5 to 13.0 days (table 1). This value is equal to approximately ¼ of the mean survival time of 54 days previously reported.[1] The finding is in agreement with the Cr^{51} half-time in man and the dog of 25 to 33 days, wherein the mean r.b.c. survival times are 100 to 120 days.[3,10] In this study, the slow continuous curve (R2) has been taken to represent the relative survival time of labeled r.b.c.

TABLE 1—The Half-Time Values of 2 Chromium-51 Clearance Components and Turnover Rates of Continuous Component in Intact and Splenectomized Calves

Animal	R_1*	R_2**	K value† of R_2
INTACT CONTROLS			
1	1.5	13.0	0.053
118	0.8	10.5	0.066
121	1.0	12.0	0.058
295	0.6	12.0	0.058
296	1.0	11.2	0.062
Mean	1.0	11.7	0.059
SPLENECTOMIZED CONTROLS			
264	1.2	11.8	0.059
271‡	1.2–1.6	10.8–13.2	0.064–0.053
302	2.8	12.5	0.055
Mean	1.8	12.1	0.057

* Early rapid component, half-time clearance in days. ** Slow continuous component, half-time clearance in days. † $K = \frac{1}{t} \cdot \ln \frac{N_o}{N_t}$. ‡ Calf died while it was affected with eperythrozoonosis; cause of death not determined; refer to text for further discussion.

Splenectomized Control Animals.—Three animals were studied to investigate any alteration in r.b.c. survival in splenectomized subjects. The results are presented (table 1). Eleven days after Cr^{51} labeling, animal 302 was inoculated with Anaplasma-infected blood.

Animal 271 developed a fatal syndrome associated with an extensive eperythrozoonosis late in the trial. This made it difficult to establish the curve due to fluctuations in the hematocrit values and Cr^{51} activity. Table 1 presents the 2 possible extreme values for the animal because of this problem. Anaplasma bodies were not encountered in the r.b.c. at any time during the disease.

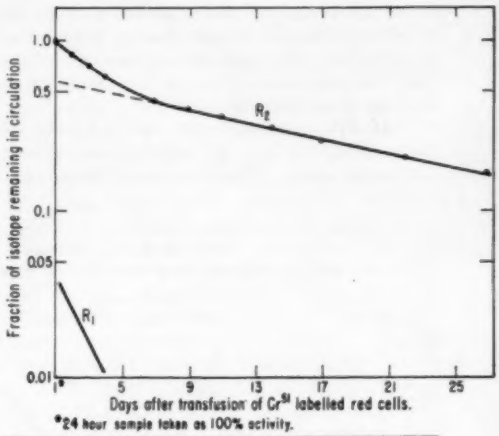

Fig. 1—Clearance of Cr^{51} labeled red blood cells from the peripheral circulation of an intact normal calf. The two components of a Cr^{51} clearance curve are shown. R_1 is an early rapid component. R_2 is assumed to represent the relative survival time of labeled red blood cells.

Animal 264 offered the opportunity to observe r.b.c. survival in a carrier animal which later underwent an anaplasmosis relapse. This heifer was assumed to be noninfected when initially studied. The complement-fixation test, however, revealed a titer of 1:10 at the time the r.b.c. were labeled. There were no Anaplasma bodies apparent in the r.b.c. at the beginning of the trial, but the animal had been held for 2½ months in an open lot among carrier cattle following splenectomy. This created the opportunity for natural infection and recovery from the initial disease process. Approximately 3 weeks after labeling with Cr^{51}, Anaplasma bodies began to appear in the r.b.c. as the animal underwent a relapse. The r.b.c. survival time then changed as described in the next section, but during the quiescent carrier state, the half-life of the labeled cells was within the normal limits.

While R2 in these animals was similar to that of intact subjects, R1 appeared to be somewhat prolonged. The significance of this has not yet been determined. In view of reports concerning different elution rates of Cr^{51} in different hemoglobins of man,[3] the possibility of altered hemoglobin in splenectomized animals might well be investigated.

Infected Splenectomized Animals.—Be-

cause the r.b.c. of these animals were labeled at different times during the course of infection, the data are presented in the text for each animal and a composite summation is presented (fig. 2).

Calf 270.—Animal 270 was infected by inoculating 15 ml. of carrier deer blood subcutaneously. Nineteen days later, antibodies began to appear and the r.b.c. were labeled on the 22nd day, near the time of anemic crisis. The Anaplasma body count was only 10 per cent and antibodies were still accumulating. The PCV was 30 per cent and the hemoglobin level was 9.0 Gm./100 ml. of blood.

Chromium-51 clearance was a single component with a half-time of 1.2 days. This corresponded to an increase in the turnover rate of 10 times normal. Because of the increased rate of R2, only 1 component was apparent. The accelerated rate was maintained until the animal died of anaplasmosis 28 days after inoculation, 6 days after isotopic labeling of r.b.c. During this period, the Anaplasma bodies reached a peak of 33 per cent 3 days before death. The count then declined to 10 per cent and the hemoglobin dropped to 2.9 Gm./100 ml. of blood.

Calf 275.—The experimental situation in this animal nearly duplicated that of animal 270. It was inoculated from the same source as animal 270 and on the same day. Antibodies began to appear after 21 days and r.b.c. were labeled on the 28th day, 6 days before the infection terminated fatally. At the time of labeling, the PCV was 26.5 per cent and there were 8.0 Gm. of hemoglobin per 100 ml. of blood. The antibody titer was still ascending and 10 to 15 per cent of the r.b.c. contained Anaplasma bodies. When the animal died, the PCV was 4 per cent; the hemoglobin level, 2.0 Gm./100 ml. of blood; and 23 per cent of the r.b.c. contained Anaplasma bodies. The antibody titer had increased fourfold (titer of 1:640) following the labeling and the Anaplasma bodies reached a peak count of 53 per cent 3 days before death. As in animal 270, the clearance curve consisted of 1 component and the half-time was 1.4 days.

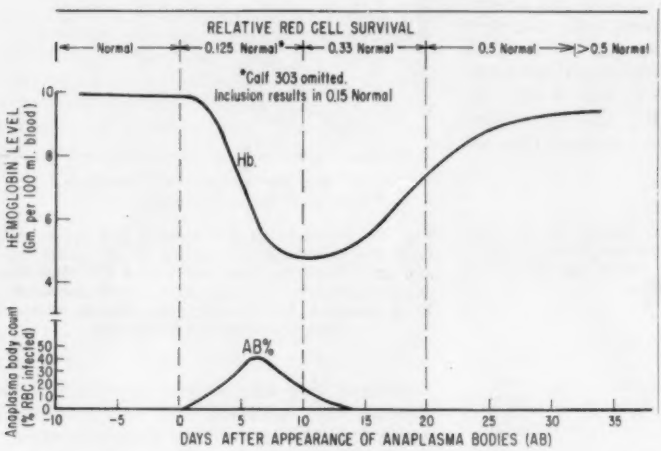

Fig. 2—Composite data from Anaplasma-infected splenectomized calves. The effect on red blood cell survival is shown in relation to phases of the anaplasmosis process.

Calf 264.—This carrier animal was discussed previously among the splenectomized control animals. The r.b.c. had been labeled and for 22 days the clearance curve was comparable to that of intact animals. There followed a lapse of 5 days before samples were again taken from the calf. A marked decrease in isotope activity was noticed between the 2 samplings. The decrease was characterized by a half-time of 1.2 days. Blood examination showed Anaplasma bodies in 10 per cent of the red cells. The hemoglobin level was 8.8 Gm./100 ml. of blood; the PCV, 28.2 per cent. These events were interpreted as a relapse of the Anaplasma infection.

Blood was relabeled on the following day and for the next 7 days a half-time

clearance of 1.4 days was determined. Following this, the slope of the curve became more gradual and no accurate conclusion could be drawn other than that the removal rate was decreasing. At this time, the complement-fixation titer was 1:80 in contrast to the titer of 1:10 that existed prior to the relapse. The decrease in turnover rate coincided with an increase in hemoglobin since the value changed from 3.2 Gm./100 ml. of blood to 6.6 Gm./100 ml. between the seventh and fifteenth days after the relabeling.

Calf 277.—Antibodies were detected 19 days after this animal was inoculated subcutaneously with 17 ml. of carrier deer blood. The r.b.c. were labeled 3 days later as the magnitude of the process was increasing. There was a hemoglobin level of 8.0 Gm./100 ml. of blood and a PCV of 28.0 per cent, with 2 to 10 per cent of the r.b.c. containing Anaplasma bodies. For 9 days, the half-time clearance was 1.4 days. The apex of the process was reached during that period. The hemoglobin level dropped to 2.8 Gm./100 ml. of blood 7 days after labeling and rose again to 4.0 Gm./100 ml. of blood on the ninth day. Anaplasma bodies reached a peak of 50 per cent 5 days after labeling and then dropped to less than 1 per cent by the ninth day.

Red blood cells were relabeled 15 days after antibodies had originally appeared. At that time the hemoglobin level had increased to 5.3 Gm./100 ml. of blood and the complement-fixation titer had reached a peak of 1:2,560. The half-time clearance for the following 8 days was 4.0 days. Then there was demonstrated the cyclic re-emergence of Anaplasma bodies that not uncommonly occurs in this disease in splenectomized calves. The period of 9 to 15 days after the second labeling was characterized by a new increase in r.b.c. removal with a half-time clearance of 1.6 days. During that period, the hemoglobin dropped from 6.8 to 5.0 Gm./100 ml. of blood and the Anaplasma body count rose to 18 per cent.

The r.b.c. were labeled a third time 35 days after antibodies had appeared at the beginning of the process. The animal was recovering from the relapse but 3 to 11 per cent of its r.b.c. still contained Anaplasma bodies. The half-time clearance during that period could not be accurately determined because of the continuing decrease in the removal rate. The lowest possible half-time, however, was in excess of 4 days, representing a turnover rate of nearly 3 times normal.

Calf 302.—The r.b.c. of this animal were labeled before it was infected with *A. marginale*. At that time, the r.b.c. volume was found to be 2,000 ml. and the total blood volume 6,500 ml. The half-time clearance rate for this normal animal is recorded (table 1). Eleven days after labeling of r.b.c., the animal was inoculated with 25 ml. of bovine carrier blood intravenously and 25 ml. subcutaneously. For the next 11 days, during the incubation period, the clearance rate remained normal. On the eleventh day, a complement-fixation titer of 1:20 had developed and the following day 1 to 2 per cent of the r.b.c. contained Anaplasma bodies. The hemoglobin level at that time was 8.7 Gm./100 ml. of blood and the PCV was 31 per cent. This represented an early stage in the process and during the next 4 to 5 days the rate of removal of r.b.c. was an increasing function with an average relative half-time of 6 days. This was in contrast to previous animals in which the clearance rates appeared to change more precipitously. Six days after the complement-fixing antibodies were first noticed, the r.b.c. containing Anaplasma bodies represented 40 per cent of the count but the hemoglobin level had only dropped to 7.0 Gm./100 ml. of blood. Over the following 4 days, the clearance rate of Cr^{51} was markedly increased and reached a half-time of 1.2 days. The apex of the Anaplasma process was reached during that period, wherein the Anaplasma body count rose to 52 per cent and the hemoglobin dropped to 3.0 Gm./100 ml. of blood. Two days after this period, the Anaplasma body count had dropped to 2 per cent.

In this animal, the increase in Anaplasma bodies had been gradual and the period during which a high percentage of cells were involved was more prolonged. This corresponded to a more gradual decline in hemoglobin and an increase of only twofold in Cr^{51} clearance. During the period of the anemic crisis, however, the clearance rate was comparable to that of other animals.

Ten days after first producing a recordable antibody response, the Cr^{51} activity had fallen to a level where relabeling was necessary. Labeled cells from normal calf 303 were transfused into animal 302. The

procedure was of limited value because the blood of the 2 appeared to be incompatible and the half-time clearance was only 0.3 days. The blood volume at this time was found to be 6,400 ml., indicating a maintenance of blood volume, since 6,500 ml. of blood had been calculated for this animal prior to infection. Of considerable interest is the fact that the blood volume was maintained even though there had been a 63 per cent decrease in hemoglobin level and a 65 per cent loss in r.b.c. volume.

Calf 303.—Infected blood from calf 302 was inoculated intravenously into this animal by transfusing 150 ml. of newly labeled cells. The transfused blood had a hemoglobin level of 3.6 Gm./100 ml., a PCV of 10.5 per cent, a complement-fixing antibody titer of 1:1,280, and an Anaplasma body count of 47 per cent. During the following 4 days, the half-time clearance was 1.8 days. Between the fourth and fifth days, the clearance rate increased and a half-time clearance of 0.3 day resulted. This resembled the clearance rate observed immediately in animal 302 when it received normal blood from this animal and is presumably the result of the development of isoantibodies against the donor cells.

An enormous *A. marginale* inoculum had been given this calf. Six days later, antibodies began to appear and after another 2 days the animal's own r.b.c. were labeled with Cr^{51}. The Anaplasma body count was only 1 per cent and there were 9.6 Gm. of hemoglobin per 100 ml. of blood at this time. The disease process was not severe and the peak Anaplasma body count of 29 per cent was reached 8 days after the appearance of antibodies. This decreased to 1 per cent by the seventeenth day. Hemoglobin values reached their lowest levels of 5.6 Gm./100 ml. of blood on the eleventh day following the appearance of antibodies. During the 15 days following labeling, the half-time clearance was 3.2 days.

The cells were relabeled on the 23rd day after antibodies appeared. The Anaplasma body count had then declined to 1 per cent and there were 8.0 Gm. of hemoglobin per 100 ml. of blood. A typical 2-component clearance curve was found with a K value of 0.08 for R2 which continued for a period of at least 18 more days.

On each of the 3 occasions, when isotopic chromium was administered to this animal, r.b.c. and total blood volume determinations were made. Consecutive values for r.b.c. volume were 3,400 ml., 3,200 ml., and 2,800 ml. Corresponding values for total blood volume were 9,700 ml., 9,600 ml., and 11,000 ml. These data again indicate the relative stability of the total blood volume in anaplasmosis.

DISCUSSION

From this study, the following generalizations can be made regarding the turnover rate of Cr^{51}-labeled r.b.c. in anaplasmosis. During the incubation period, the rate is apparently normal, as indicated by animal 302. The rate changes within 1 to 4 days following the appearance of Anaplasma bodies in r.b.c. In 5 of 6 animals in this study, the rate increased 8 to 10 times normal. In 1 instance (calf 303), the increase was only 3.6 times. The degree of anemia, never severe in this animal, correlated with the smaller increase in turnover rate. Hemoglobin levels in animals which survived the infection began to increase when the turnover rates decreased to 3 to 4 times normal. During recovery, the rates continued to decrease, but for periods as long as 40 days (animals 277 and 303) after the initial crisis, rates were still 1.5 to 2.0 times normal.

The data suggest that there is a limiting factor which determines the maximum r.b.c. removal rate encountered in anaplasmosis and that this factor is similar in most animals. The maximum rate appears to lie between 8.0 and 10.0 times normal, and it is noticed that even during the relapse of animal 277, a rate of 7.4 times normal prevailed. This same maximal rate appears to occur regardless of the number of r.b.c. visibly parasitized. It is tempting to suggest that this limiting factor may be the size and potential of the reticuloendothelial system functioning at a maximal rate in its catabolism of r.b.c.

The fact that donor cells were removed at a much greater rate (animals 302 and 303) than homologous Anaplasma-infected cells lends support to the existence of this limiting factor, the basis of this support being that a much smaller number of cells in the recipient animals are destined for removal and thus the reticuloendothelial system would not become "saturated." Another possible explanation for the rapid removal of donor cells might rest in their

agglutination in the peripheral vascular bed with subsequent retention and lysis in capillaries.

The above observations offer the interesting possibility that successful blockade of the reticuloendothelial system may have therapeutic value in the disease. In this respect, the prophylactic value of cortisone on the relapse in carrier animals subjected to splenectomy by Ristic and Sippel [11] is of interest.

The lack of correlation between the rate of removal and the number of cells with Anaplasma bodies is likewise of interest. When r.b.c. were labeled prior to the appearance of Anaplasma bodies or when the parasitized cell count was only 2 to 10 per cent, the turnover rate was constant throughout the period of decreasing hemoglobin values, even though the numbers of cells with Anaplasma bodies increased from 0 to 50 per cent and then decreased to 10 to 30 per cent. In addition, the rate was similar in different animals regardless of whether they reached peak Anaplasma body counts of 30 or 50 per cent. This has several implications. With regard to the pathogenesis of the disease, the foregoing would indicate that, if only r.b.c. which contain Anaplasma bodies are subject to early removal, the "organism" must enter the r.b.c. after the latter has entered the peripheral circulation. If this removal is dependent upon a specific antibody, then the antigen-antibody combination must also occur in the circulatory system. This is true since only the pool of r.b.c. which is circulating at the time of labeling is marked with Cr^{51}. As noted, the r.b.c. in animal 302, labeled prior to infection, were removed during the anemic crisis at the same rate as those labeled in other animals (270, 275) during the anemic crisis. This same observation was made in the carrier animal (264) which relapsed subsequent to labeling of the r.b.c.

As a second implication, if only those cells containing Anaplasma bodies are removed at an increased rate, then this portion of the total r.b.c. population must be assumed to surpass the previously considered limiting factor. That this is possible may be indirectly surmised. If one assumes the conservative value of 50 days [1] as being normal for the mean r.b.c. survival time in calves, it is apparent that 2 per cent of the r.b.c. would be removed daily. An increase of 8 times this value would require that 16 per cent of the cells contain the disease agent. Since the rate of removal in most animals was of this magnitude when only 2 to 10 per cent of the circulating r.b.c. contained Anaplasma bodies, the fact that r.b.c. removal is a continuous dynamic function and not a once-a-day event must be considered. If removal of 2 to 10 per cent of the r.b.c. population, in our hypothetical case, is integrated over a period of 24-hours, in time elements of less than 3 hours, the total number of r.b.c. removed in the 24-hour period would exceed 16 per cent. Red blood cells containing Anaplasma bodies in the peripheral circulation may actually represent an accumulated excess above the maximum capacity for removal. Other possible explanations which fit the present data in this respect are that many more cells are marked for early destruction than indicated by the presence of visible Anaplasma bodies or that some unrecognized immunologic event such as the production of auto-antibodies against the homologous r.b.c. may take place. The former possibility seems particularly feasible in view of the electron microscopic demonstration of submicroscopic disease agents in Anaplasma-infected red cells.[7]

With regard to the "gross" mechanism of the anemia, it has been demonstrated in swine [5] that hemopoiesis can be increased only 4 to 5 times. If a similar limitation on hemopoiesis applies to cattle, it is apparent that the increased turnover rates observed in this study would readily produce a marked anemia. Since a given amount of functional marrow tissue can produce only a relatively fixed amount of hemoglobin in a given time, the compensatory response of an animal undergoing a hemolytic crisis in anaplasmosis would be slow until additional amounts of yellow marrow could be converted to red marrow. The probable importance of this relationship was exemplified in calf 277. In that animal, a severe decrease in hemoglobin occurred during the primary hemolytic crises. During a subsequent relapse of similar duration and turnover rate, however, only a small decrease in hemoglobin level occurred. This might well be attributed to a greater amount of functional marrow at the time of relapse.

Further uncertainty of the significance of bone marrow depression in anaplasmosis

may be derived hypothetically. Assuming that there is little change in blood volume during the course of the disease (calves 302 and 303) and that calves normally remove a maximum of 2 per cent of the circulating hemoglobin daily, it is apparent that complete inhibition of hemopoiesis would account for a decrease in the hemoglobin level of only 20 per cent over a 10-day period. The actual decrease observed in this disease approximates 60 to 80 per cent.

It is concluded that the major factor in the production of anemia in anaplasmosis is acute extravascular hemolysis and, if bone marrow depression occurs, its influence is, in all likelihood, of little or no significance.

SUMMARY

By the use of Cr^{51}-labeled red blood cells (r.b.c.) it was found that the r.b.c. survival during the hemolytic crisis in splenectomized calves infected with *Anaplasma marginale* is reduced 8- to 10-fold. Since animals appear to be able to increase their production of hemoglobin by only 4 to 6 times, it is apparent that this increased destruction of r.b.c. would result in marked anemia.

On the basis of the turnover rate of r.b.c., it is hypothesized that a "limiting factor" is operative in the maximal rate at which r.b.c. are removed in anaplasmosis. It is suggested that this factor is the maximal rate at which the reticuloendothelial system may function in the catabolism of r.b.c.

Experimental data as well as hypothetical calculations are presented which indicate that the anemia in this disease is primarily, if not entirely, due to hemolysis.

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SUMMARIO IN INTERLINGUA

Le Superviventia de Erythrocytos in Anaplasmosis Experimental

Per le uso de erythrocytos marcate con Cr^{51}, il esseva trovate que le superviventia erythrocytic durante le crise hemolytic in vitellos splenectomisate que es inficite con *Anaplasma marginale* es reducite a inter un octavo e un decimo. Viste que le animales es capace a augmentar lor production de hemoglobina per solmente 4 a 6 vices, il es evidente que iste augmentate destruction de erythrocytos debe resultar in marcate grados de anemia.

Super le base del metabolismo de erythrocytos, le hypothese es formulate que un "factor limitante" es active in determinar le mesura maximal a que erythrocytos es eliminate in anaplasmosis. Es proponite que iste factor es le maximo al qual le systema reticuloendothelial pote functionar in le catabolismo de erythrocytos.

Es presentate datos experimental e etiam calculationes hypothetic que indica que le anemia in iste morbo es primarimente, si non totalmente, le consequentia de hemolyse.

Comparison of the Flotation of Metastrongylus and Ascaris Eggs in 3 Different Levitation Solutions

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MANY WORKERS have studied the comparative efficiency of various flotation techniques for the recovery of parasite eggs and cysts from feces. Various flotation agents, such as sodium chloride, zinc sulfate, sodium nitrate, sucrose, cupric nitrate, and calcium chloride have been used.[1, 3-5, 7-14] The results of these tests indicate that the flotation efficiency of a given solution will vary, depending on the particular species of eggs in the fecal sample.

Dunn and White [3] and Dunn [1] compared sodium chloride and magnesium sulfate solutions of a specific gravity of 1.200 and found that the magnesium sulfate recovered *Metastrongylus apri* eggs from feces that had been found to be negative for these eggs by sodium chloride flotation. No explanation was offered for these results. In an earlier report, Wilson [14] attempted to examine the factors he considered to influence the difference in the flotation efficiency of cane sugar and sodium chloride. The 3 factors considered were the relative specific gravity, the viscosity, and the rate of plasmolysis caused by the solutions.

The objectives of the present work were to compare the flotation efficiency of magnesium sulfate, sodium chloride, and sucrose solutions in recovering eggs of Metastrongylus spp. and *Ascaris lumbricoides*; to confirm the results of Dunn and White [3]; and to offer an explanation for the differences usually found in the number of eggs recovered from the same fecal sample when various flotation agents were employed.

MATERIALS AND METHODS

Fecal samples were collected from 4 pigs on a farm where there was a history of Metastrongylus infections. Four other samples were collected from pigs known to be infected with *A. lumbricoides*. Each sample was well mixed to insure that the portion selected for experimentation would accurately represent the sample.

A 2-Gm. sample of feces was mixed with 20 ml. of distilled water in a beaker and poured through a 40-mesh wire sieve into two 15-ml. centrifuge tubes. The beaker was then washed with an additional 10 ml. of distilled water, and this, too, was poured over the material remaining on the sieve. The tubes were centrifuged at 1,500 r.p.m. for 3 minutes, and most of the supernatant fluid was aspirated, leaving a small layer of the fluid over the undisturbed residue. The residue was mixed with the flotation agent which was added until a small positive meniscus was formed at the top of the tube. A 22 by 22 mm. cover slip sealed the tube. The tubes were again centrifuged as before, and the eggs attached to the cover slip from each tube were counted. More flotation agent was added to again form a meniscus, and a 2nd cover slip was positioned. The specimen was centrifuged, using the same time and r.p.m., and examined. This procedure was repeated for subsequent examinations.

This method was used for both the Ascaris and Metastrongylus egg counts, except for the substitution of the different flotation agents, and the use of 0.25-Gm. samples of the feces containing Ascaris eggs. This small sample was necessary, since the numbers of Ascaris eggs in the 2-Gm. samples were so high as to introduce errors in counting. Magnesium sulfate, sodium chloride, and sucrose (with 0.6% phenol added) solutions, standardized by a hydrometer to a specific gravity of 1.200, were used as the flotation agents. In general, this method was the same as used by Dunn, *et al.*,[2] with one exception: the 2-Gm. fecal sample was mixed with 30 ml. of distilled water instead of 30 ml. of the flotation agent as advocated by these earlier workers. In preliminary experiments using the original method, the magnesium sulfate did not sufficiently dissociate the sample to pass through the sieve and release eggs. However, when the sample was mixed first with water, it became well dissociated.

RESULTS AND DISCUSSION

The number of Metastrongylus eggs counted on each of the cover slips, and the number of eggs per gram floated by the 3 flotation agents for each of the 4 fecal samples, are compared (Table 1). Generally, the number of eggs per gram recovered from the same sample was the highest with $MgSO_4$ and the lowest with NaCl; the num-

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ber floated by sucrose was between the 2 extremes.

The comparative abilities of the 3 solutions studied to float *Ascaris lumbricoides* eggs are shown (Table 2). The flotations of sample 8 are in the same order of levitation ability as was seen with the Metastrongylus eggs. However, these results are exceptional when compared with samples 9, 10, and 11. In these samples, the sucrose solution recovered the greatest number of eggs and, in samples 10 and 11, the NaCl solution floated more eggs than did the $MgSO_4$ solution. In these 2 samples, the difference between the NaCl and the $MgSO_4$ flotation was only 12 and 32 eggs per gram. When the total number of eggs counted is considered, it is doubtful that this difference is significant. The lack of equality in sample 8 may be explained, in part, by the heavy egg-load of the sample. The sodium chloride crystallized badly around the covers on the slide, and during long counts of the heavily loaded specimen it is conceivable that losses occurred by eggs being trapped in the crystals. This does not, however, explain the lower count in the sucrose solution.

The results of the Metastrongylus egg flotations confirm the observations of Dunn and White [3] that for the recovery of lungworm eggs $MgSO_4$ is more efficient than NaCl solution. Magnesium sulfate was also a consistently better agent than sucrose solution for the flotation of Metastrongylus eggs.

It was questioned whether the ability of $MgSO_4$ to float more eggs than the other solutions was a constant factor depending on the particular agent, or whether the results would be different with eggs of another species. Wilson [14] compared the flotation ability of sugar and NaCl solutions and found that eggs with heavy walls or those which were centrifuged were not seriously harmed by the NaCl, even though the calculated rate of plasmolysis by NaCl was determined to be 2.5 times that of the sugar solution. He stated that the viscosity or frictional force was not a factor affecting the time of levitation, since it is largely overcome by the application of centrifugal force.

In the present work, the factors which Wilson stated would influence the recovery of eggs were considered. The variable of specific gravity was eliminated by stand-

TABLE 1—Comparison of Various Flotation Solutions for the Detection of Metastrongylus spp. Eggs in the Feces of Swine

Flotation agent	Fecal sample No.	No. of eggs per 2 Gm. of feces, counted after intervals of 3 minutes' centrifugation at 1,500 r.p.m.				
		Cover slip 1	Cover slip 2	Cover slip 3	Total	e.p.g. recovered
$MgSO_4$	2	Tube 1–155 Tube 2– 63	1 3	1 2	225	112.5
Sucrose	2	Tube 1– 31 Tube 2– 26	0 0	0 0	57	28.5
NaCl	2	Tube 1– 0 Tube 2– 4	0 0	0 0	4	2.0
$MgSO_4$	3	Tube 1– 73 Tube 2– 34	6 6	0 1	120	60.0
Sucrose	3	Tube 1– 17 Tube 2– 6	1 1	0 0	25	12.5
NaCl	3	Tube 1– 15 Tube 2– 13	0 0	0 0	28	14.0
$MgSO_4$	6	Tube 1– 76 Tube 2– 44	6 4	0 2	132	66.0
Sucrose	6	Tube 1– 69 Tube 2– 44	0 0	0 0	113	56.5
NaCl	6	Tube 1– 0 Tube 2– 0	0 0	0 0	0	0
$MgSO_4$	7	Tube 1–149 Tube 2– 37	20 7	3 1	217	108.5
Sucrose	7	Tube 1– 74 Tube 2– 18	0 0	0 0	92	46.0
NaCl	7	Tube 1– 26 Tube 2– 18	0 0	0 0	44	22.0

TABLE 2—Comparison of Various Flotation Solutions for the Detection of Ascaris lumbricoides Eggs in the Feces of Swine

Flotation agent	Fecal sample No.	No. of eggs per 0.25 Gm. of feces, counted after intervals of 3 minutes' centrifugation at 1,500 r.p.m.											
		Cover slip 1	Cover slip 2	Cover slip 3	Cover slip 4	Cover slip 5	Cover slip 6	Cover slip 7	Cover slip 8	Cover slip 9	Cover slip 10	Total	e.p.g. recovered
$MgSO_4$	8	Tube 1– 1,672	249	103	2	0	–	–	–	–	–	2,759	11,036
		Tube 2– 534	142	38	17	2	–	–	–	–	–		
Sucrose	8	Tube 1– 1,195	590	97	17	10	1	–	–	–	–	2,243	8,972
		Tube 2– 304	21	6	2	0	0	–	–	–	–		
NaCl	8	Tube 1– 315	380	68	232	170	81	22	6	2	0	1,371	5,484
		Tube 2– 41	25	3	18	5	1	1	1	0	–		
$MgSO_4$	9	Tube 1– 766	116	68	13	11	1	–	–	–	–	1,097	4,388
		Tube 2– 100	17	4	1	0	0	–	–	–	–		
Sucrose	9	Tube 1– 775	183	26	2	–	–	–	–	–	–	1,127	4,508
		Tube 2– 124	11	4	2	–	–	–	–	–	–		
NaCl	9	Tube 1– 454	201	66	30	19	3	–	–	–	–	881	3,524
		Tube 2– 56	35	11	6	0	–	–	–	–	–		
$MgSO_4$	10	Tube 1– 110	43	31	10	3	0	–	–	–	–	271	1,084
		Tube 2– 63	6	4	1	0	–	–	–	–	–		
Sucrose	10	Tube 1– 331	11	1	–	–	–	–	–	–	–	406	1,624
		Tube 2– 46	17	0	–	–	–	–	–	–	–		
NaCl	10	Tube 1– 113	94	24	7	10	0	–	–	–	–	279	1,116
		Tube 2– 17	8	6	0	–	–	–	–	–	–		
$MgSO_4$	11	Tube 1– 222	32	17	5	0	–	–	–	–	–	307	1,228
		Tube 2– 21	5	4	1	–	–	–	–	–	–		
Sucrose	11	Tube 1– 224	86	7	2	–	–	–	–	–	–	364	1,456
		Tube 2– 41	4	0	0	–	–	–	–	–	–		
NaCl	11	Tube 1– 107	58	35	11	9	6	0	–	–	–	310	1,240
		Tube 2– 57	17	9	1	0	–	–	–	–	–		

ardizing all solutions to sp.g. 1.200. The effects of viscosity were considered eliminated by centrifuging the samples at 1,500 r.p.m. for 3 minutes. By comparing the recovery of the relatively thick-walled Ascaris eggs by $MgSO_4$, sucrose, and NaCl solutions, the effect of the rate of plasmolysis on the more thin-shelled Metastrongylus eggs became more apparent.

McDonald [6] found that NaCl solution affected Pneumoneces and Clonorchis eggs by causing the operculum to separate from the rest of the egg shell. This he attributed to "either osmotic pressure or the variation in pressure between the medium and the contents of the ovum."

In later work, Sawitz [8] found a difference in the yield of whipworm eggs from NaCl and $ZnSO_4$ solutions of the same specific gravity. This he considered as evidence of a possible interaction between the eggs and the levitation medium, the $ZnSO_4$ apparently interfering less, possibly by virtue of the difficulty of the sulfate ion in penetrating the living cell membranes.

The results (Table 2) indicate that, apparently, with the Ascaris eggs there was less interference by the medium; with the specific gravity of the agents as a constant, the number of eggs floated by the solutions from any one sample was close. The results from sample 8 were the only exceptions. This might be due to crystallization obscuring eggs and to possible sampling error.

A comparison of the levitation agents shows NaCl solution to be the least desirable for the purpose of diagnosis by flotation, due to the rapid crystallization that the medium undergoes on the cover slip, thus obscuring a large area of the slide and causing shrinkage of the film. Possibly, the lower counts with the NaCl agent were due to the crystals, concealing many of the eggs from view. As a medium for the diagnosis of Metastrongylus infections, NaCl should not be used due to the apparently great interaction with the eggs.

Sucrose solution proved to be quite effective for the recovery of Ascaris eggs, but generally was a poor second to $MgSO_4$ as a flotation agent for Metastrongylus eggs. Films on slides prepared with sucrose solution did not crystallize or shrink. However, mold readily grew in the stock solution, even with phenol added, and the sucrose attracted insects.

The $MgSO_4$ solution at sp.g. 1.200 proved to be the best agent for the recovery of Metastrongylus eggs and an effective agent for the levitation of Ascaris eggs. Slight crystallization occurred around the edge of the cover slip and sealed it to the slide, preventing further crystallization. At a higher specific gravity (1.300), $MgSO_4$ was not desirable, since a greater amount of debris was floated, obscuring many of the eggs.

SUMMARY AND CONCLUSIONS

The relative flotation ability of $MgSO_4$, sucrose, and NaCl solutions at sp.g. 1.200 for the recovery of Metastrongylus and Ascaris eggs from swine feces was determined by a modification of the centrifugation-flotation method used by Dunn *et al.*[2]

It was found that the NaCl solution was ineffective for the diagnosis of Metastrongylus eggs, and that certain physical characteristics of the medium itself made it the least desirable method for the recovery of Ascaris eggs.

Sucrose solution recovered the highest number of Ascaris eggs, but was not as good as $MgSO_4$ for floating Metastrongylus eggs.

The best agent studied for the levitation of Metastrongylus and Ascaris eggs was $MgSO_4$ solution at sp.g. 1.200; it apparently interfered less with the eggs than did the other solutions. The use of NaCl or sucrose greatly lessened the chance of recovering Metastrongylus eggs.

It is suggested that the difference in the numbers of eggs recovered by the 3 flotation agents was due to the effects of osmosis or to the differences in the rates of plasmolysis caused by the solutions.

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SUMMARIO IN INTERLINGUA

Comparation del Flottation de Ovos de Metastrongylus e de Ascaris in Tres Differente Solutiones de Levitation

Le relative capacitate flottatori de $MgSO_4$, sucrosa, e NaCl in solutiones de un gravitate specific de 1,200 in le constatation de ovos de Metastrongylus e de Ascaris in le feces de porcos esseva determinate per un modification del methodo de centrifugation e flottation usate per Dunn *et al.*[2]

Esseva trovate que le solution de NaCl esseva inefficace in le diagnose de ovos de Metastrongylus e que certe characteristicas physic del medio mesme rendeva lo le minus desirabile in le diagnose de ovos de Ascaris.

Le solution de sucrosa revelava le plus alte numero de ovos de Ascaris, sed illo esseva minus bon que $MgSO_4$ pro le flottation de ovos de Metastrongylus.

Le melior del agentes studiate pro le levitation de ovos de Metastrongylus e Ascaris esseva un solution de $MgSO_4$ al gravitate specific 1,200. Illo apparentemente interfereva minus con le ovos que le altere solutiones. Le uso de NaCl o de sucrosa reduceva grandemente le probabilitate del recovration de ovos de Metastrongylus.

Es proponite le explication que le differentias in le numeros del ovos recovrate per le tres agentes de flottation esseva le effecto de osmosis o de differentias in le intensitate del plasmolyse inducite per le solutiones.

Effect of Naturally Occurring Highly Mineralized Water on the Growth of Laboratory Animals

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FOR MANY YEARS, laboratories have been called upon to analyze certain well waters and to pass judgment as to whether such waters were harmful to animals. Most of such judgments have been based on limited observations, and in only a few cases has experimental evidence been presented to support them.

Animals from certain feedlots in the north central part of Colorado, supplied with highly mineralized water obtained from shallow wells, have not developed properly during the fattening period. Sickness and death have been attributed to water from shallow wells; some supposed sickness was believed to have diminished as a result of changing to water of lesser mineral content.

This study was an attempt to learn whether highly mineralized water affects the growth rate of laboratory animals.

REVIEW OF LITERATURE

A review of the literature concerning the effects of the consumption of highly mineralized water on human beings and animals permits a number of generalizations and implications:

1) Flavor generally prevents human beings and animals from consuming excessive amounts of highly mineralized water.

2) Harmful effects [2, 6–7, 17, 18, 21, 23, 29, 30, 31] may be direct (due to action of the solutes) or indirect (from dehydration due to the lack of consumption of sufficient water).

3) Intestinal disturbances [29] encountered in changing from one water supply to another are probably due to adjustments of the intestinal tract to new metabolic conditions, especially in pH.

4) Animals [6, 18, 24] can adjust to highly mineralized waters. Injurious effects are demonstrated by sudden changes from one water supply to another without a period of adjustment. Tolerance appears to increase in man and animals with continued consumption of highly mineralized water. Ruminants appear to have more tolerance for such water than do nonruminants.

5) Ruminants [2, 5, 10, 14, 26, 19] appear to be susceptible to nitrate poisoning from water; whereas, nonruminants do not appear to be susceptible under normal conditions.

6) Infants [1, 4, 8, 9, 12, 22, 25, 28] appear to be more susceptible to methemoglobinemia because the pH of the gastric juice of infants normally is higher than that of normal adults.

7) Nitrates [20] that enter shallow wells need not always be attributed to nearby sources of pollution.

8) Public water supplies [11, 25, 27] should be examined at frequent intervals. Mineral constituents in water may vary with the season but are more apt to do so in times of prolonged drought or excessive rainfall.

MATERIALS AND METHODS

For this study, 157 male white mice, 27 female guinea pigs, and 33 castrated male rabbits were observed for 3 months. All of the animals were earmarked for identification upon receipt, and individually weighed. Using a frequency-distribution table of the weight classes, animals were allotted to experimental and control cages, alternately. Eight cages were used for the mice, 2 for the guinea pigs, and 4 for the rabbits. The self-waterers and feeders fastened to the cages were constructed so as to minimize waste.

Water was obtained from 2 sources. The term "naturally occurring highly mineralized water" is applied to water obtained from a well on the farm owned by J. L. Templin, Eaton, Colo. The water contained a high concentration of mineral salts (Table 1). The term "Fort Collins water" is applied to water obtained from a water hydrant in the Colorado State University Veterinary Hospital. This water contained a low concentration of mineral salts (Table 1).

The water was collected in U.S. Army water cans lined with baked-on enamel and fitted with snap-type lids. It was stored in a refrigerator until time of use. Blue and red tags were permanently attached to the cans to identify each water source. The water was analyzed at 10-day intervals. Pelleted feed was used during the entire period (Table 5).

The balance scale used to weigh the mice had a 0.1-Gm. accuracy; that used for weighing the guinea pigs and rabbits had a 1.0-Gm. accuracy. Each mouse was confined in a plastic food con-

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TABLE 1—Average Findings from Analyses of the Two Types of Water Given Free-Choice to Experimental Animals

Content	Highly mineralized water (p.p.m.)	Fort Collins water (p.p.m.)
Total solids	9,725.8	52.0
Calcium sulfate ($CaSO_4$)	2,328.8	.....
Magnesium sulfate ($MgSO_4$)	3,947.4	.....
Sodium sulfate (Na_2SO_4)	895.0	.....
Sodium nitrate ($NaNO_3$)	236.4	.....
Sodium chloride (NaCl)	279.9	.....
Sodium carbonate (Na_2CO_3)	546.2	.....
Potassium carbonate (K_2CO_3)	9.4	.....
Fecal and organic	74.7	trace
Volatile matter	1,408.0	24.0
Reaction (pH)	7.4	6.9
Bases		
Calcium oxide	949.4	trace
Magnesium oxide	1,333.7	trace
Sodium oxide	1,297.8	present
Acids		
Nitrates	159.0	.....
Chlorides	169.8	.....
Sulfates	4,469.2	.....
Bicarbonates	261.5	slight

Chemical analyses by Joseph W. Tobiska (deceased), and Dale D. Maag, Colorado State University.

tainer for weighing; each guinea pig and each rabbit was held in a galvanized wire cage.

The average daily gains were used as the variates for comparison. The average for each animal was obtained by subtracting the original weight from the final weight and dividing the number of treatment days (91 days for mice, 94 for guinea pigs, and 90 for rabbits).

The design of the experiment made possible a "nested" or hierarchical classification of the sources of variation for analysis. Water from each source was supplied to 4 cages of mice, 1 cage of guinea pigs, and 2 cages of rabbits. The analyses of variances were performed separately for the 3 kinds of laboratory animals, and were used as the tests for significant differences in average daily gains.

RESULTS

The *F*-tests of significance indicate that the rates of gain for rabbits and mice were not significantly different between those supplied with highly mineralized water and those supplied with Fort Collins water.

The guinea pigs supplied with Fort Collins water made significantly higher rates of gain in body weight than did the guinea pigs supplied with mineralized water.

The analyses of variances are shown (Table 2).

DISCUSSION

Review of the literature did not reveal any instance in which data obtained from animals consuming highly mineralized water in this region had been analyzed statistically. Neither was a large number of experimental animals, together with control animals, subjected to tests.

In our experiment, no attempt was made to determine effects on the animals other than changes in weight. These changes in weight were used as the variates upon which the analyses of this study have been based (Table 3). Average daily consumption of water is shown for information only and was not used as part of the pertinent data (Table 4).

If an animal finds water unpalatable, it seems unlikely that it would consume sufficient amounts to cause physical harm from the chemical constituents. Negus [23] reported that it probably requires over 100 p.p.m. of lime hydrate to produce a distinct, lime-like flavor in water. Magnesium salts will affect the taste of water even more than the calcium ions. Both calcium and magnesium salts are known to be essential to proper body functions, and it seems inconceivable that an animal would consume enough unpalatable water containing these salts to cause injurious effects.

The consumption of highly mineralized water has been considered from 2 aspects: (1) the direct effect of the solutes upon the animal and (2) the indirect effect,

TABLE 2—Analyses of Variances for the Average Daily Gains of Laboratory Animals Given Two Types of Water

Sources of variation	Degrees of freedom			Mean square		
	Mice	Rabbits	Guinea pigs	Mice	Rabbits	Guinea pigs
Water	1	1	1	0.002295	19.455	1.7225*
Cages in water groups	6	2	..	0.001644	13.538	.....
Animals in cages	149	29	25	0.002389	6.310	0.273291

* Significance $F > 0.05, < 0.01$.

resulting from the lack of sufficient water to carry on the normal body processes. Heller,[16] in 1933, reported that a number of these solutes, when used in excessively large amounts, produced ill effects, retardation of growth, and toxic conditions in various animals. The salts of sodium and magnesium sulfates, sodium carbonate, sodium bicarbonate, and sodium chloride occur in the greatest amounts. It seems probable that the combinations of bicarbonates in water have more injurious effects than do combinations of the other salts. Combinations of bicarbonates and carbonates react with the hydrochloric acid in the stomach and reduce the acidity of the gastric juice, which then cannot take various elements into solution for utilization within the body; this indirectly results in digestive disturbances. The osmotic effect of some salts causes injury. Rapid loss in weight, resulting from severe diarrhea caused by magnesium and sodium sulfate administration, has been seen in all animals.

TABLE 3—Average Daily Weight Gain in Grams per Animal

Species	Highly mineralized water	Fort Collins water
Mice	0.136	0.129
Guinea pigs	2.887	3.393
Rabbits	19.055	17.519

Intestinal disturbances have been reported in human beings after a sudden change in water supply. Such disturbances have disappeared after using the water for a period of time, as was the case with water of the San Luis Valley of Colorado. An adjustment of the body to the pH changes created in the intestinal tract may explain the rapid recovery. If these sudden changes could be avoided by a gradual transition from one water to another, these intestinal disturbances might be avoided.

TABLE 4—Average Daily Consumption in Grams

Species	Highly mineralized water		Fort Collins water	
	Water	Food	Water	Food
Mice	8.982	6.429	8.204	6.380
Guinea pigs	90.263	38.385	76.000	38.234
Rabbits	.....	173.275	.....	169.094

TABLE 5—Analyses of Composite Samples of Pellets Given Free Choice to Experimental Animals

Per cent by weight*	Purina Rabbit Pellets	Rockland Farms Guinea Pig Pellets	Purina Fox Chow Pellets (fed to mice)
Total ash	10.6900	9.875	8.716
Calcium oxide	4.1200	2.200	3.040
Magnesium oxide	0.0362	0.159	0.115
Phosphoric oxide	0.0241	0.015	0.030

Chemical analyses by Dale D. Maag and Joseph W. Tobiska (deceased), Colorado State University.
* Other components were not analyzed.

Heller[16] reported that ruminants appear to have more tolerance for highly mineralized water than do nonruminants. This may be due to the abundant bacterial flora found in the rumen. The contents of the ruminant stomach are exposed to the action of bacteria longer than the contents of the nonruminant stomach. This may provide an opportunity for detoxification of harmful chemicals by bacteria before the chemicals reach the absorptive areas of the intestines. The fact that methemoglobinemia occurs only in infants with a low acidity of the gastric juice was reported by Cornblath and Hartmann.[9] This may explain why ruminants, because of the high pH of the rumen, are susceptible to methemoglobinemia; whereas, it appears that nonruminants are not susceptible to methemoglobinemia because of the low pH in their simple stomach.

If this is the case, it may account for the failure to obtain significant differences in the growth of laboratory animals in the present study. All laboratory animals used in this experiment were animals with simple stomachs that presumably contained gastric juices of high acidity. It might be of interest in a future study to attempt to produce methemoglobinemia in a nonruminant animal by isolating a loop of the small intestine to form a Thiry-Vella fistula, as described by Markowitz,[20] and infusing sodium nitrate and nitrate-reducing bacteria into the loop.

From the records at Colorado State University, it has been found that many wells contain water with a high concentration of nitrates. Tobiska[26] believed that nitrates in soil may be leached by rain waters and carried into the shallow wells. Periods of drought seem to concentrate nitrate in the soil and account for higher accumulation of nitrates in water in shallow wells during such periods. In 1954, when water was

collected for this experiment, northern Colorado was extremely dry. This may have accounted for the high concentration of nitrates found in the water obtained from the Eaton well. If this is the case, it seems desirable to examine wells at frequent intervals, particularly in periods of drought.

The analyses of variances were conducted to test the differences between the average daily weight gains of animals consuming highly mineralized water and those consuming Fort Collins water. Though every effort was made to keep conditions constant for animals in both groups, slight differences may have been due partly to differences in cage conditions beyond our control. The *F*-tests gave only weak evidence of real differences in the effects of water.

SUMMARY

Young rabbits, mice, and guinea pigs were allotted to 2 groups. Over a period of 3 months, those in one group were supplied with water having a high mineral content; those in the other group were supplied with water containing a low concentration of mineral salts.

The average daily weight gains of the animals in the 2 groups were used as the variates for comparison of the effects of the 2 kinds of water.

The guinea pigs supplied with water having a low mineral content made significantly higher rates of gain in body weight than did guinea pigs supplied with highly mineralized water.

Rates of gain for rabbits and mice were not significantly different.

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SUMMARIO IN INTERLINGUA

Le Effecto de Altemente Mineralisate Aqua Super le Crescentia de Animales de Laboratorio

Juvene conilios, muses, e porcos de India esseva distribuite in duo gruppos. Durante un periodo de tres menses, le animales in un del gruppos recipeva aqua a alte contento mineral; le animales in le altere del duo gruppos recipeva aqua a basse concentrationes de sales mineral.

Le augmentos medie de peso per die in le animales del duo gruppos esseva usate como variatos pro le comparation del effectos del duo generes de aqua.

Le porcos de India mantenite con aqua a basse contento mineral se distingueva per significativemente plus marcate augmentos del peso corporee ab le porcos de India mantenite con altemente mineralisate aqua.

Le augmentos de peso non differeva significativemente in le caso de conilios e muses.

Metastrongylosis in the Field: Species and Sex Ratios of the Parasites, Preferential Location in Respiratory Apparatus of the Host, and Concomitant Lesions

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OF THE 3 species of swine lungworms reported in the United States, *Metastrongylus apri* is considered the most prevalent, *Metastrongylus pudendotectus* is next in abundance, and *Metastrongylus salmi* is the least common.[9]

In general, *M. apri* is more abundant in swine lungworm populations, though certain workers have reported situations in which *M. salmi* was predominant.[5, 8]

Spindler reported a 69% prevalence in lungworms in southern swine and found *Metastrongylus elongatus (M. apri), Choerostrongylus pudendotectus (M. pudendotectus),* and *M. salmi* in 69, 50, and 12%, respectively, of the animals examined.[10] Only the females were counted, and they ranged in numbers per infected animal as follows: 1 to 167, 1 to 97, and 1 to 18 (in the same order as above). Hawkins found a 31% prevalence among Michigan swine.[9] Goldsby and Todd reported a 56% prevalence among Wisconsin market-weight swine.[6] The latter 2 reports gave neither the species nor sex ratios, and none of the 3 reports specified the location of the helminths in the respiratory apparatus or the gross lesions concomitant with the parasites.

The present study details the relative abundance of the various metastrongyle species and the sex ratios of these parasites among 100 naturally infected midwestern swine. A correlation of the gross lesions concomitant with these helminthiases also is presented.

MATERIALS AND METHODS

The lungs and tracheas of 100 market-weight swine infected with lungworms were collected at a Madison, Wis., packing house. The material was collected over a period of a year at frequent, though irregular, intervals.

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Respiratory organs were brought to the laboratory and given an identification number, and the gross lesions were recorded.

An effort was made to obtain all worms without mutilation. Magnifying eyepieces were worn during dissection to insure recovery of the small male helminths.

The trachea was removed by making a small incision between 2 of the cartilaginous rings and then extending the incision so as not to rupture worms which might be present. The trachea then was opened from the larynx to its bifurcation.

The intermediate lobe of the right lung was then removed, and small scissors were employed to open the bronchial tree to its termination. This procedure was repeated with the right apical, right cardiac, right diaphragmatic, left apical-cardiac, and left diaphragmatic lobes, in that order.

Each helminth recovered was examined microscopically, and its species and sex was determined.

RESULTS

The 3 species of Metastrongylus reported in the United States were all found to be present in swine marketed in Wisconsin. Both males and females of all 3 species were recovered.

TABLE 1—Summary of Helmiths Removed from 100 Swine Naturally Affected with Metastrongylosis

Species and sex	No. recovered	Sex distribution (%)
Metastrongylus apri, male	1,222	36.5
Metastrongylus apri, female	2,130	63.5
Subtotal	3,352	
Metastrongylus pudendotectus, male	890	44.0
Metastrongylus pudendotectus, female	1,129	56.0
Subtotal	2,019	
Metastrongylus salmi, male	25	50.0
Metastrongylus salmi, female	25	50.0
Subtotal	50	
Total, males	2,137	39.4
Total, females	3,284	60.6
Grand total	5,421	

The species and sex ratios of the helminths recovered in the 100 swine are summarized (Table 1). *Metastrongylus apri,* the most prevalent, constituted 61.8% of the total

TABLE 2—Numbers of Lungworms Found in Various Parts of Respiratory Systems of Naturally Infected Swine

Species and sex	Right lobes					Left lobes		Total
	Apical	Cardiac	Diaphragmatic	Intermediate	Trachea	Apical-cardiac	Diaphragmatic	
M. apri, male	37	15	620	24	4	18	504	1,222
M. apri, female	51	13	1,062	41	9	44	910	2,130
M. pudendotectus, male	15	6	456	11	0	14	388	890
M. pudendotectus, female	19	12	551	24	1	7	515	1,129
M. salmi, male	1	0	9	0	0	0	15	25
M. salmi, female	1	0	7	0	0	0	17	25
Total	124	46	2,705	100	14	83	2,349	5,421

metastrongyles recovered; *M. pudendotectus* made up 37.2% and *M. salmi* the remaining 1%. There was some variation in the sex ratios within the 3 species; the general ratio of all females to all males was approximately 3:2 (60.6: 39.4%).

The preferential location of the helminths is shown (Table 2). The diaphragmatic lobes were the most heavily infected; the right one contained 50% of all helminths recovered, and the left one contained 43%. The remaining 7% of worms were distributed, in order of decreasing numbers, in the right apical, intermediate, left apical-cardiac, and right cardiac lobes and the trachea. The frequency with which the given locations were parasitized is shown (Table 3).

A wide variation in the magnitude of infection is obvious from the range in numbers of the various species and sexes in the several lobes and the trachea (Table 4).

In the distribution of the 3 species, the prominent feature is the frequent association of *M. apri* and *M. pudendotectus* (Table 5).

Emphysema and chronic inflammatory responses were the most constant gross lesions observed. The gross lesions (Table 6) observed in the 100 swine with field cases of metastrongylosis and the concomitance of helminthiasis and these lesions are indicated (Tables 7, 8). A definite correlation seems to exist between presence of helminths in the lungs and the presence of emphysematous lesions. Emphysema was

TABLE 3—Percentage of Locations in Swine Respiratory Apparatus which Contained at Least 1 Lungworm

Species and sex	Right lobes				Trachea	Left lobes	
	Apical	Cardiac	Diaphragmatic	Intermediate		Apical-cardiac	Diaphragmatic
M. apri, male	11	4	72	8	4	7	63
M. apri, female	10	5	78	12	8	12	72
M. pudendotectus, male	4	3	63	5	0	7	54
M. pudendotectus, female	4	3	65	5	1	4	60
M. salmi, male	1	0	6	0	0	0	9
M. salmi, female	1	0	4	0	0	0	6

TABLE 4—Range of Numbers of Lungworms Recovered in Specific Locations of Naturally Infected Swine

Species and sex	Right lobes				Trachea	Left lobes	
	Apical	Cardiac	Diaphragmatic	Intermediate		Apical-cardiac	Diaphragmatic
M. apri, male	0–14	0–9	0–86	0–8	0–1	0–6	0–56
M. apri, female	0–15	0–7	0–88	0–12	0–2	0–9	0–63
M. pudendotectus, male	0–8	0–3	0–39	0–4	0	0–3	0–44
M. pudendotectus, female	0–9	0–8	0–43	0–11	0–1	0–3	0–42
M. salmi, male	0–1	0	0–4	0	0	0	0–3
M. salmi, female	0–1	0	0–4	0	0	0	0–4

present in approximately 90% of the lobes which contained helminths. No tracheal lesions were observed.

DISCUSSION

Wisconsin market-weight swine were found to harbor all 3 species of Metastrongylus reported in the United States and, judging from this study, the general sex ratio in this genus is 3:2, favoring the female. The individual species had some variation; only in *M. salmi*, however, was this exceptional. This could be due to the limited series of helminths available for observation. In all 3 species, there was considerable variation in the ratio within a given host animal. Indeed, some animals were found to be parasitized by a single sex of only 1 species. Obviously, these were rare and exceptional situations.

TABLE 5—Per Cent of Naturally Infected Swine Harboring a Single Species Versus Multiple Species of Swine Lungworms

Species	Per cent of total
Metastrongylus apri	11
Metastrongylus pudendotectus	1
Metastrongylus salmi	1
M. apri and *M. pudendotectus*	76
M. apri and *M. salmi*	0
M. pudendotectus and *M. salmi*	0
All 3 species	11

The tendency of metastrongyles to parasitize the diaphragmatic lobes of the lungs in preference to other lobes was confirmed in this study. Since 93% of the total helminths recovered came from these 2 lobes, the tendency seems well established. There seems to be no differential preference for any given location by the various species or by the sexes of any 1 species.

TABLE 6—Gross Respiratory Lesions Observed in 100 Swine Naturally Affected with Metastrongylosis

Lesion observed	No. of lobes
Atelectasis	4
Chronic inflammatory response	270
Emphysema	234
No gross pathologic changes	199
Total No. lobes examined	600

The magnitude of the infections was quite varied. Factors of husbandry practices, acquired immunity, age resistance, and the "self-cure" phenomenon, as they affect levels of metastrongylosis, remain to be studied.

TABLE 7—Concomitance of Helminthiasis and Gross Lesions in Swine Naturally Affected with Metastrongylosis

Detail	Number
Lobes with lesions	401
With helminths	210
Without helminths	191
Lobes without lesions	199
With helminths	5
Without helminths	194
Total No. lobes examined	600

Lesions associated with the presence of metastrongyles were mainly emphysema and chronic inflammatory responses. Although inflammatory responses were more frequently observed than was emphysema, the former was often seen in the absence of helminths. To divorce the chronic inflammatory lesions from the helminthiasis seems unjustified, but to attribute them to the helminths would be presumptuous. The inflammatory lesions were more often observed in the proximal lobes; the distal lobes (diaphragmatic) harbored most of the helminths and were extensively emphysematous. The chronic inflammatory response was often seen in these distal lobes both in the presence and absence of emphysema. The cause of the chronic inflammatory response, then, was obscure; it is possible that many of these lesions were those of virus pneumonia of pigs.[1-4] Emphysema, however, seemed to result from stenosis of the air passages by the helminths. The lobes with emphysema in the absence of helminths could have lost the parasites, or the lesions could have been produced by a different cause.

TABLE 8—Concomitance of Helminthiasis and Specific Gross Lesions in Swine Naturally Affected with Metastrongylosis

Lesion observed	Number of lobes		
	Free of helminths	Concomitant with helminths	Total
Atelectasis	1	3	4
Chronic inflammatory response	167	103	270
Emphysema	41	193	234

The concomitance of the chronic inflammatory response with metastrongylosis could be coincidence or an etiologic com-

plex. The metastrongyles conceivably could act as an initial invader and be followed by bacterial or viral opportunists, or both. The true significance of these helminths in this chronic inflammatory response is a matter of conjecture. If an association does exist, then the role of the helminth as primary or secondary remains to be determined.

Summary and Conclusions

Data obtained during a study of 100 swine with field cases of metastrongylosis indicated that *Metastrongylus apri, Metastrongylus pudendotectus,* and *Metastrongylus salmi* occurred in the following approximate relative abundance: 62, 37, and 1%, respectively, of the total worms recovered; females outnumbered males approximately 3:2. In 76% of the infections, *M. apri* and *M. pudendotectus* occurred in association; in 11%, 3 species were present; and single species were involved in the remaining 13%.

The preferential location of metastrongyles in the respiratory apparatus was established as being in the diaphragmatic lobes. There was no tendency for any given species or sex to parasitize a specific area of the respiratory apparatus.

Gross lesions concomitant with metastrongylosis were mainly emphysema and chronic inflammatory responses. Emphysema appeared to be characteristic of lungworm infection, but the cause of the chronic inflammatory response could not be established from this study.

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Summario in Interlingua

Metastrongylosis in le Campo: Proportiones del Distribution Secundo Species e Sexos, Location Preferential in le Apparato Respiratori del Hospite, e le Lesiones Occurrente in Concomitantia

Datos obtenite durante un studio de cento porcos con metastrongylosis de campo indicava que *Metastrongylus apri, M. pudendotectus,* e *M. salmi* occurreva approximativemente con le sequente abundantia relative: 62, 37, e 1 pro cento, respectivemente, del numero total de vermes retrovate; femininas excedeva masculos in un proportion de approximativemente 3:2. In 76 pro cento del infectiones, *M. apri* e *M. pudendotectus* occurreva in association; in 11 pro cento, tres species esseva presente; species individual esseva interessate in le remanente 13 pro cento.

Le location preferential de metastrongylos intra le apparato repiratori esseva le lobos diaphragmatic. Esseva constatate nulle tendentia de un specie particular o del un o del altere del sexos de concentrar se specificamente in un area particular del apparato respiratori.

Lesiones macroscopic occurrente in concomitantia con metastrongylosis esseva primarimente emphysema e chronic responsas inflammatori. Emphysema pareva esser characteristic de infection per vermes pulmonar, sed le causa del responsa inflammatori chronic non poteva esser establite a base del presente studio.

The Recovery of *Bacterium anitratum* (B5w) from Animals

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No REPORTS of the recovery of *Bacterium anitratum* (B5w) from animals have appeared in the literature, but it has been a suspected pathogen for man since about 1948. Beginning with a chance isolation from chickens, interest has been aroused concerning the possible role of *Bact. anitratum* in animal disease. Because the organism does not fit into most schemes of identification, no doubt it has been discarded many times as a nonpathogen and has gone unidentified. It is the purpose here to report limited isolations of *Bact. anitratum* from animals. Included in the discussion is a review of the literature, which is concerned entirely with human infections. The organism is characterized biochemically and human strains are compared with those from animals.

Since DeBord[2,3] described a new tribe of bacteria called Mimeae, many investigators[1,2,5,9] have found in human lesions organisms biochemically and serologically compatible with DeBord's cultures. DeBord would place *Bact. anitratum* in the genus Herellea which he discovered while investigating cultures of Neisseria-like organisms suspected of being *Neisseria gonorrhea*. These organisms, because of their striking similarity to the Neisseria, were easily mistaken in smears. DeBord reached the conclusion that the diagnosis of gonorrhea by smears was not justified, due to this possible error.

For several years, Stuart and Formal[8] had isolated an unidentifiable gram-negative organism which appeared to occupy a position between the genuses Proteus and Shigella. They isolated it from the blood of patients with high fevers and from 4 persons with diarrhea. It was most often isolated from urine and feces but differed from Proteus in its failure to attack urea, to produce indol, or to break down nitrates to nitrites. The organism utilized citrate readily. One per cent lactose was not fermented, but 10% lactose slants were readily fermented. Evidence indicated that a new group of organisms had been discovered. It was designated B5w for convenience (Institution B, 5th culture, white colony). The numerous cultures were found to be antigenically homogenous.

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Schaub and Hauber[7] reported an unidentifiable gram-negative organism resembling the Neisseriae. It was isolated from various pathologic processes. In smears, it appeared as a diplococcus. A large number of isolations were made chiefly from urine, although occasionally from sputum, lung abscesses, and blood. The morphologic and biochemical characteristics of the organisms were described in detail. The name "*Bacterium anitratum*" seemed appropriate due to apparent inability to reduce nitrates to nitrites. Differentiation was made from Shigella by serum-agglutination reactions. It was also shown that intraperitoneal injection of strains of *Bact. anitratum* were pathogenic for mice and guinea pigs.

While studying capsule formation, Schaub and Hauber discovered that all naturally occurring strains possessed a capsule with which could be demonstrated the "quellung" reaction, using a rabbit serum. R and S forms would not agglutinate when this serum was used. Tests showed that when the organisms were in the natural or mucoid phase, they were homogenous and were not serologically associated with gram-negative bacilli of the Enterobacteriaceae or any other family.

Biochemical and serologic studies have since been carried out by Ewing,[4] Ferguson and Roberts,[5] Brooke,[1] and Waage.[9] These authors characterized the bacterium as a nonmotile, gram-negative encapsulated coccoid bacillus with simple biochemical requirements. Sugar fermentations were reported as variable, but generally the organism formed acid but no gas from glucose, arabinose, and xylose. It was methyl red and Voges-Proskauer negative, citrate positive, indol negative, H_2S negative, gelatin negative, and negative for nitrate reduction. A facultative aerobe, it formed neither spores nor pigment and was oxidase negative. Hemolytic strains were found. The feature of fermenting lactose in 10% slants, but not in 1% broth, was characteristic. Upon initial isolation, it could easily be mistaken morphologically for Pasteurella sp. Ferguson and Roberts[5] have isolated the organism from 40 urine and 23 sputum specimens and also from 1 fecal specimen. Brooke[1] isolated 56 strains of the bacterium from urine specimens, abscesses, and sputum.

Brooke,[1] during a study of Enterobacteriaceae in urine, sputum, secretions, and abscesses, reported an "unusual gram-negative coccobacillary organism." The description given compares favorably with that of other workers for *Bact. anitratum*. Of 86 strains studied, 11 were tested for pathogenicity in mice. Intraperitoneal injection of 0.2 cc. of an 18-hour broth culture proved fatal with 3 strains. Eight strains proved apathogenic.

The apathogenicity of some cultures is in agreement with other investigators. It has been shown by several workers that pathogenicity for laboratory animals is variable and that not many strains are pathogenic at all. Only the intraperitoneal route has proved fatal for mice or guinea pigs.

Waage [9] reported a case wherein a patient, following trauma to the back and hip, became semicomatose and developed a high fever. Neurologic examination indicated injury to the brain. At autopsy, it was found that 3 cerebral abscesses were present on the left side. The organism isolated proved to be *Bact. anitratum*. Its biochemical reactions were similar to those of organisms isolated by several American workers,[4,5,8] and to animal strains reported herein.

CASE REPORTS

In the course of routine diagnosis of avian specimens submitted to the Department of Animal Diseases, Georgia Coastal Plain Experiment Station, a small gram-negative organism was recovered from the livers of several of a group of chickens. The birds were 14 months of age and were layers. Vaccination had been completed against bronchitis, Newcastle disease, and pox. At 8 weeks of age, the birds in the flock of 4,000 contracted cecal coccidiosis and were apparently treated successfully with sulfaquinoxyline. The birds were making normal gains when, at the age of 14 months, they began to die. Early mortality resulted in the loss of approximately 10% of the flock. On culture, the livers of several of the birds were found to contain an organism that was gram-negative, with a tendency toward bipolar staining. It had the following biochemical characteristics: glucose and xylose were fermented with the formation of acid but no gas; mannitol, lactose, sucrose, and maltose were not fermented; 10% glucose and lactose slants were fermented. The organism grew on MacConkey agar and SS agar. It was citrate positive, nitrate negative, indol negative, H_2S negative, methyl red negative, and Voges-Proskauer negative. Litmus milk showed an acid curd. The organism did not form spores, was oxidase negative, and produced *beta* hemolysis on ox blood sugar. It was nonmotile. Although pasteurella-like in morphology, it did not conform to the biochemical classification of this genus and was, therefore, referred to Dr. P. R. Edwards at Chamblee, Ga., who identified it as *Bact. anitratum*.

On Sept. 13, 1959, 6 live Leghorn chickens were received at the Animal Disease Diagnostic Labortary of the Florida Livestock Board, Kissimmee, Fla. The owner stated that he had lost approximately 50 in a flock of 1,500 over a period of about 2 weeks. In the past 3 days, 30 had died. Necropsy of the chickens revealed numerous petechial hemorrhages on the mucous membrane of the small intestine and fewer on the serosal surface. The spleens were enlarged and some livers were mottled. White foci were observed on the surface of several spleens. The carcasses were yellowish with a few hemorrhages visible in the leg muscles of some birds. The mean packed cell volume was 22%. The birds were negative for parasites. A Streptococcus and a gram-negative bipolar rod were isolated from the spleens and livers of 5 of the 6 birds examined. The latter differed from the first *Bact. anitratum* isolate in that it was not hemolytic on ox blood agar. Results of biochemical examination were otherwise almost identical to the initial isolate (Table 1).

The third isolation of *Bact. anitratum* was made from a calf found dead in a pasture. The swollen head gave the immediate impression of blackleg or snake bite and was delivered to the laboratory for examination. Incision of the skin over the face revealed a dark bloody fluid. The musculature was hemorrhagic and dark and no gas was noticed. Microscopic examination of this material and other samples from various areas of the head did not reveal any organisms morphologically suggesting Clostridium sp. Routine anaerobic cultures were made and a tissue suspension was injected intramuscularly into a guinea pig. Six mice were given 0.3 ml. of tissue suspension intravenously to rule out snake venom. Of 2 guinea pigs inoculated intraperitoneally with 3 cc. of tissue fluid, 1 died in approximately 8 hours and the other in 12 to 14 hours. The mice inoculated intravenously remained without clinical signs. Six mice inoculated intraperitoneally died in approximately 12 hours. On postmortem examination, the guinea pigs had no apparent gross lesions, with the exception of peritonitis. The livers of these guinea pigs were cultured in heart infusion broth and a gram-negative coccoid rod was isolated that had the biochemical characteristics associated with *Bact. anitratum*. A diagnosis of snake bite or clostridial infection could not be substantiated.

TABLE 1—Biochemical Reactions of Bacterium anitratum Isolates

Strain	Glucose	Xylose	Mannitol	Lactose	Sucrose	Maltose	10% Lac.	1% Glucose	MacConkey	SS	Citrate	Urea	Nitrate	Indol	H_2S	Methyl Red	V.P.	Gelatin	Lit. milk	Morph.	Gram	Spores	Use of oxygen	Pigment	Oxidase	Hemolysis	Motility	Catalase
1972	A	A	−	−	−	−	A	A	+	−	+	−	−	−	−	−	−	+	−	coc.	−	−	Facultative	−	−	−	−	4+
11208	A	A	−	−	−	−	A	A	+	+	+	−	−	−	−	−	−	+	AC	coc.	−	−	Aerobic	−	−	Beta	−	2+
2409	A	A	−	A^7	−	−	A	A	+	−	+	−	−	−	−	−	−	+	AC	coc.	−	−	Facultative	−	−	Beta	−	4+

A = acid; A^7 = acid in 7 days; AC = acid curd.

DISCUSSION

Bacterium anitratum was found in predominating numbers from the livers of chickens in 2 separate epizootics and from fluid obtained from the swollen head of a calf. The importance of streptococci in the organs of chickens was not known in either instance. No other bacteria were isolated from the fluid removed from the calf head. This strain proved pathogenic for both mice and guinea pigs when injected intraperitoneally.

Investigation of available literature and personal communication with Dr. W. W. Ferguson, Michigan Department of Health, would appear to confirm the impression that this organism has not been reported from animal hosts.

Just how important *Bact. anitratum* may become in animal disease processes can only be surmised. If its involvement in human disease is to be taken as an index, it may be well to look for this bacterium in animals. Because *Bact. anitratum* has appeared to be associated with Proteus, Klebsiella, coliform organisms, nonhemolytic streptococci, or staphylococci,[1,5,8,9] the possibility of not recognizing it would seem good.

Bacterium anitratum (B5w) bears some relationship to the coliform group, but failure to reduce nitrate may exclude it from the Enterobacteriaceae. Ewing[4] compared *Bact. anitratum* to the tribe Mimeae, described by DeBord, and would place the organism in this tribe. Henriksen[6] has pointed out that the tribe Mimeae includes unrelated organisms though they may appear morphologically similar.

Stuart *et al.*[8] isolated *Bact. anitratum* from feces cultured anaerobically. They believed the organism should, therefore, be classified as an anaerobe. Animal strains isolated thus far grow only aerobically. It is suggested that animal strains be classified in the genus Bacterium due to their inability to reduce nitrate. The tribe Mimeae has been placed in Parvobacteriaceae and does not appear to include *Bact. anitratum* as pointed out by Henriksen.[6]

Bacterium anitratum can easily be identified by its fermentation of 10% lactose in slants and by its failure to ferment 1% lactose in broth. Carbohydrates are fermented only aerobically, the fermentation in a glucose Durham tube occurring in the outer tube only. These observations, coupled with finding a short, plump, gram-negative

rod showing a tendency toward bipolar staining, should make identification easy. It is believed that the isolations herein reported should be called to the attention of veterinary bacteriologists.

SUMMARY

A gram-negative bipolar rod isolated from chickens and a calf was classified biochemically as *Bacterium anitratum* (B5w). Isolations were made from chickens and from the head of a calf having, at necropsy, signs of snake bite or Clostridium infection. Intraperitoneal inoculation of one isolate (strain 2409, Table 1) proved fatal for guinea pigs and mice.

The 3 strains isolated differed only in their hemolytic activity on 5% ox blood agar and the ability of 1 strain (2409) to ferment lactose in 7 days. Slight differences were noted in ability to use oxygen. Studies indicated that the organism may be pathogenic for animals.

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SUMMARIO IN INTERLINGUA

Le Recovration de *Bacterium anitratum* (B5w) ab Animales

Un baculo bipolar e negative al Gram que esseva isolate ab gallinas e un vitello esseva classificate biochimicamente como *Bacterium anitratum* (B5w). Isolationes esseva effectuate ab gallinas e ab le capite de un vitello que exhibiva al necropsia signos de un morsura de serpente o de infection per Clostridium. Le inoculation intraperitonee de un isolato (racia 2409, Tabula 1) se provava letal pro porcos de India e pro muses.

Le tres racias isolate differeva solmente in lor activitate hemolytic in agar a 5 pro cento de sanguine bovin e in le facto que un del racias (2409) esseva capace a fermentar lactosa intra septe dies. Leve differentias esseva notate in le capacitate de usar oxygeno. Varie studios indicava que le organismo es possibilemente pathogene pro animales.

Scientific Notes

Cytologic Changes Induced by Infectious Canine Hepatitis Virus in Tissue Culture

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THE RELATIONSHIP of infectious canine hepatitis virus to adenoviruses of human origin has been demonstrated.[5] One criterion was the marked similarity of cytopathogenicity in tissue culture of the 2 virus types. This similarity is obvious in comparing the studies on infectious canine hepatitis (ICH) virus[7] with those on adenovirus.[2, 5, 9] This report presents additional data on the morphology of intranuclear changes in ICH virus-infected cells and further discusses the relationship of the virus to the adenoviruses.

Materials and Methods

Tissue Culture.—Virus was grown in cultures of canine kidney cells. The cells were removed from fragments of kidney cortices of dogs less than 6 months of age, using 0.25% trypsin solution in a 250-ml. trypsinizing flask (Bellco). Trypsinization was done for approximately 18 hours at 5 C. Cells were suspended in medium 199 with 10% horse serum, and planted in Leighton tubes containing coverglasses. Cell sheets were usually confluent in about 7 days, and at this time were used for virus seeding.

Virus.—The 61st passage of an attenuated virus, obtained originally from Dr. James A. Baker, Cornell University, was used. Coverglasses with kidney tissue were removed from the Leighton tubes at 12, 24, 30, 36, 48, and 72 hours after infection, fixed, stained, and examined.

Staining.—Kidney tissues on coverglasses were stained with Giemsa stain or hematoxylin and eosin. Methyl alcohol was the fixative in the former, and Bouin's solution was used for the hematoxylin and eosin procedure.

Results

Infected cells first had a margination of basophilic nuclear chromatin with some adjacent rarefaction of the nucleus. Enlargement of the nucleus was not obvious early in infection (Fig. 1).

The central portions of the nuclei then became well-defined inclusion bodies, stained more densely, and filled approximately ¾ of each nucleus. This corresponds to the stage II changes described previously (Fig. 2).[7]

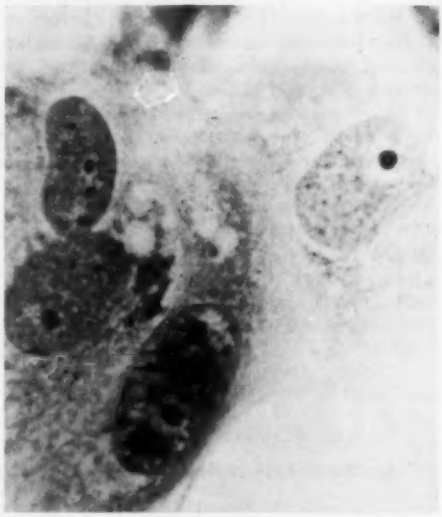

Fig. 1—Early stage of inclusion body development following infection of canine renal epithelial monolayer with infectious canine hepatitis virus. Giemsa stain; x 700

As the inclusion body developed, strands of chromatin were present, extending from the inclusion body to the periphery of the nucleus. Some increase in size of the nucleus became obvious, and the nuclear membrane became irregular, producing a lobular effect (Fig. 3).

Inclusions in advanced development had a definite internal architecture (Fig. 4, 5). A number of irregularly shaped, eosinophilic, homogeneous bodies appeared to be imbedded in the inclusion matrix. These bodies were present in cells in advanced stages of degeneration.

The total number of detectable infected cells in the sheet increased slowly through 48 hours,

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Fig. 2—Canine renal epithelium infected with infectious canine hepatitis virus showing well-defined inclusion body with rarefaction of the nucleus and some chromatin strand formation. Giemsa stain; x 700.

Fig. 3—Chromatin strands extending from the inclusion body to the periphery of the nucleus and lobular appearance of the nuclear membrane following infection of canine renal epithelium with infectious canine hepatitis virus. Giemsa stain; x 700.

Fig. 4—Well-defined bodies within the advanced inclusion body of infectious canine hepatitis infected renal epithelium. Giemsa stain; x 700.

approximating results reported previously.[7] Between 24 and 48 hours, nearly all stages of cell change were detectable in any slide.

Discussion

The striking similarity between the development of the ICH inclusion body and that of adenovirus in tissue culture has been emphasized.[5] The results of the present study further confirm this close similarity. One point of difference was noticed previously, however, in regard to the crystalline structures associated with the adenovirus inclusions.[5] These bodies, shown to be virus accumulations, had not been noticed in ICH inclusion bodies. In the present study, homogeneous structures within the inclusions strongly suggest that crystalline virus aggregates may exist. Electron microscopic studies of ICH virus[12] did not demonstrate the presence of such crystals, but the studies were done on infected dog liver cells rather than on cells in tissue culture.

It is of further interest that the canine and human viruses retain a rather strict host-cell speci-

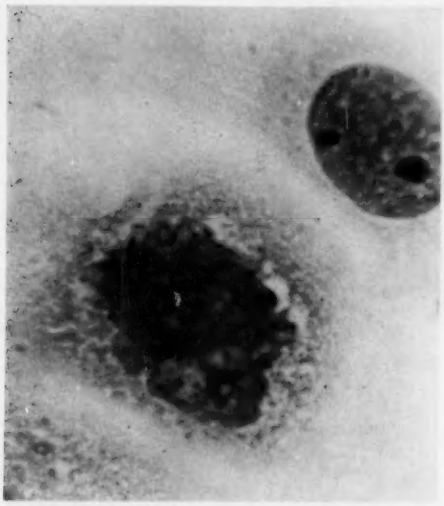

Fig. 5—Nucleus of canine renal epithelial cell in advanced stage of infection with infectious canine hepatitis virus, showing multiple irregularly shaped areas within the inclusion mass. Giemsa stain; x 700.

ficity. In spite of the identity of cellular lesions produced, adenovirus produces these changes in cells of primate origin and ICH virus in canine cells, but neither agent is heterotrophic for these cell types.

The following relationships exist between adenovirus and infectious canine hepatitis virus: (1) group serologic identity,[6] (2) identity of cellular pathology, (3) intranuclear site of virus multiplication,[1, 2, 12] (4) lack of infectivity for laboratory animals,[5] (5) establishment of a temporary carrier state in the natural hosts of both agents, (6) similarities of some aspects of the natural disease produced by both viruses, and (7) same particle size of intranuclear viruses.[1, 12]

There appears to be no reason for hesitation in admitting the close relationship of the 2 viruses. It is proposed, therefore, that ICH virus be definitely classified as an adenovirus. Until more specific serologic data become available, ICH cannot be placed with the numbered sequence of known adenoviruses. For purposes of clarity, it is suggested that a new group, adenovirus Group B, be established to include, in subgroupings, the adenoviruses of nonprimate origin. Thus, the following classification might be derived:

Adenovirus Group B—viruses of animal origin:
- Type 1—Infectious canine hepatitis virus
- Type 2—Bovine viruses[4]
- Type 3—Avian viruses (tentative)[4]

The suggestions that ICH virus may have some public health significance[10] or that adenoviruses may be transmissible from man to dogs[11] have been made. In the light of present knowledge, it certainly would be essential that virus isolation with specific typing for identification be done in order to give confidence to these suggestions.

Summary

During this study of the cytologic changes induced by infectious canine hepatitis virus in tissue culture, special attention was give to cellular lesions in canine renal epithelium, development of the inclusion body, and similarities to lesions produced by adenoviruses.

Homogeneous structures noticed within the inclusion body were similar to those found in adenovirus-infected cells, which suggest the presence of crystalline virus aggregates. The relationships between human adenovirus and infectious canine hepatitis virus were reviewed and it was suggested that a new group be established, for classification purposes, to include all adenovirus agents other than those of primate origin.

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SUMMARIO IN INTERLINGUA

Alterationes Cytologic Inducite in Histoculturas per Virus de Hepatitis Infectiose Canin

In le curso de iste studio del effectos cytologic inducite per virus de hepatitis infectiose canin in histoculturas, attention special esseva prestate a lesiones cellular in canin epithelio renal, le disveloppamento de corpores de inclusion, e similaritatos con lesiones producite per adenoviruses.

Structuras homogenee trovate intra le corpore de inclusion esseva simile a illos trovate in cellulas inficite de adenovirus, lo que suggere le presentia de crystallin aggregatos de virus. Le relation inter adenovirus human e virus de hepatitis infectiose canin es revistate. Es proponite le establimento de un nove gruppo—pro objectivos de classification—que includerea omne agentes adenovirusal excepte illos de origine in primates.

The Effect of Urinary Silicon Concentration on the Formation of Siliceous Deposits on Bladder Implants in Steers

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ZINC implantation in the urinary bladders of experimental animals is used as a means of inducing urinary calculi formation. Under appropriate experimental conditions, the technique has been successfully used in rats for the formation of phosphate-type stones [3] as well as in the formation of stones of high silicon content.[4]

The experimental work in this study was undertaken to ascertain if the feeding of a ration high in silicon alone would be sufficient to cause the formation of artificial siliceous urinary stones on bladder implants in steers. The results reported suggest that enhanced urinary excretion of silicon may not be the only factor involved in the formation of siliceous calculi in the bovine species.

Materials and Methods

Two separate implant experiments were performed on steers to examine the effects of various dietary regimens on the formation of deposits on the implants.

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In the first experiment, 4 Hereford steers, 7 to 8 months of age and weighing 300 to 350 lb., were used. Feed was withheld for 48 hours and water was withheld for 24 hours prior to implant surgery. At the time of surgery, each steer was given an injection of a tranquilizer intravenously and 10 to 20 cc. of 2% procaine hydrochloride solution epidurally, then cast and restrained in dorsal recumbency. The skin of the ventral abdomen was shaved and scrubbed and an antiseptic was applied. The incision site was infiltrated with 2% procaine. Starting just anterior to the pubis and extending anteriorly, a paramedian incision, 5 inches long, was made through the abdominal wall 1½ inches from the midline, care being taken to avoid the penis. The bladder could then be easily located, but bringing it to the incision was sometimes difficult.

In the first experiment, each steer was implanted with a piece of mossy zinc, a piece of smooth sheet zinc, and a siliceous urinary stone which had been previously removed from a steer affected with clinical calculosis. The zinc pellets and stone were tied together with silk suture then sutured to the mucosa of the floor of the bladder. Each implant had been accurately weighed, the weights being approximately 1.5 Gm. for the mossy zinc, 2.0 Gm. for the sheet zinc, and 0.15

Gm. for the urinary stone. Two of the steers were given a high silicon, low water ration composed of 100 Gm. of $Na_2SiO_3 \cdot 9H_2O$ freshly dissolved in water with the pH adjusted to 10.8 with HCl immediately before giving it to the steer in the daily ration of 6 liters of water. This represents nearly the maximum dose of $Na_2SiO_3 \cdot 9H_2O$, prepared in this way, which a steer can tolerate because of the NaCl resulting from neutralization of the metasilicate. The other 2 steers served as implanted controls and were given water free choice. Urine was collected from the steers for 8- or 24-hour periods for silicon analysis.[4] The treatments were continued for about 60 days. The implants were surgically removed approximately 20 days after treatments were stopped. The steers were fed alfalfa hay during the first 35 days of treatment and grass hay for the remainder of the experiment.

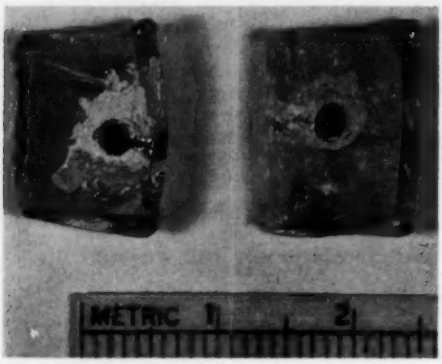

Fig. 1—Implants after removal from steers in experiment 2.

In the second experiment, two 1-year-old steers were implanted with pieces of sheet zinc and were fed 300 Gm. of freshly ground SiO_2 per steer per day suspended in a daily ration of 7 liters of water. It was found that a more greatly enhanced urinary silicon excretion could be achieved in that way than through metasilicate administration. Furthermore, SiO_2 is the chemical form of most of the silicon of plant material.[6] The treatment was continued for 25 days. These 2 steers were given grass hay throughout the experiment. The implants were removed 7 days after treatment was terminated.

Results and Discussion

In the first experiment, the steers on silicon supplementation excreted 41.0 γ/ml. (s.d. = 7.0) of silicon in the urine while being fed alfalfa hay, and 96.5 γ/ml. (s.d. = 19.0) of silicon in the urine while on grass hay. The control steers excreted silicon in the urine at a level of 18.5 γ/ml. (s.d. = 3.0) while on alfalfa hay and 74 γ/ml. (s.d. = 19.0) on grass hay. Examination of the implants after removal revealed that no material was deposited on any of the 12 implants from control or treated steers.

In the second experiment, the 2 steers excreted silicon at an average level of 189 γ/ml. (s.d. = 69.0) during the 25 days they were given silicon. At the termination of the experiment, the steers were slaughtered, the urinary tracts examined, and the zinc pellets removed and weighed. In both steers, there was only a trace of deposit found on the pellets (Fig. 1). One had increased in weight by 7.9 mg., and the other by 19.9 mg. No abnormalities were found upon examination of the urinary tracts except for a trace of gritty material in the renal pelvis of 1 steer.

The steers given dietary additions of silicon were excreting it at a level much higher than the 70–80 γ/ml. level at which silicon precipitates in bovine urine.[7] This was especially true in experiment 2 and true at least some of the time during the second part of experiment 1 when the steers were given grass hay.

The lack of pronounced siliceous deposits suggests the involvement of other factor(s) in the cause of urolithiasis besides a simple high concentration of urinary silicon. The most obvious possibility is, of course, the mucoprotein found in bovine stones.[3, 5] The mucoprotein component found in urinary stones of various species has received attention as a possible agent in the cause of urolithiasis.[1, 2, 8] It may be that the urinary level in steers in the present study was not sufficiently high to result in the formation of concretions. Experiment 2 was performed in May and June. These are warmer months than the November-to-February period, during which most cases of bovine siliceous calculi are reported in this area. While no determination of urinary mucoprotein constituents was performed on the 2 steers used in experiment 2, urinary hexose concentrations were determined on 2 similar steers in late June and found to be much lower than the averages[7] for steers in the winter months. A determination of total concentration of an arbitrarily selected constituent from urinary mucoprotein may or may not reflect a change in concentration of a specific mucoprotein involved in stone formation.

It would seem desirable to investigate the influence of still higher levels of urinary silicon on the formation of deposits on bladder implants, particularly in the winter months when the urinary mucoprotein levels may be relatively high. It would be profitable to determine if any correlation exists between high urinary silicon-high urinary muco-

protein levels and the formation of deposits on the implants. Experiments are being planned to investigate these aspects of the problem.

Summary

A dietary regimen for enhancing urinary silicon excretion in steers is described. The excretion level achieved was two- to threefold greater than the 70 to 80 γ/ml. level at which silicon normally precipitates in bovine urine. Steers having bladder implants and excreting as high as 189.0 γ/ml. (s.d. = 69.0) of urinary silicon failed to show appreciable siliceous deposit on the implants after 25 to 60 days on the regimen. The possibility is considered that high urinary silicon is not the only factor involved in the formation of siliceous calculi in the bovine species.

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SUMMARIO IN INTERLINGUA

Le Effecto de Concentration Urinari de Silicium Super le Formation de Depositos Silicee in Implantas Vesical de Tauros

Es describite un regime dietari visante a promover le excretion urinari de silicium in tauros. Le nivello de excretion effectuate esseva inter duo e tres vices le nivello de 70 a 80 gamma per ml al qual silicium es normalmente precipitate in urina bovin. Tauros con implantas vesical e excretiones de silicium urinari de usque a 189,0 gamma per ml (d.s. = 69,0) non monstrava appreciabile depositos silicee in le implantas post 25 a 60 dies de subsistentia con le mentionate regime. Es considerate le possibilitate que alte nivellos de silicium urinari non es le sol factor in le formation de calculos silicee in le specie bovin.

Meetings • • •

• • • World Meeting on Experimental Surgery. Turin, June, 1961. Minerva Medica, Corso Bramante 83-85, Turin, Italy.

• • • World Symposium on Genetics. Turin, June, 1961. Minerva Medica, Corso Bramante 83-85, Turin, Italy.

• • • World Symposium on the Physiopathology of Renal Papilla. Turin, June, 1961. Minerva Medica, Corso Bramante 83-85, Turin, Italy.

• • • International Congress of Rural Medicine. Tours, France, July 2-9, 1961. Prof. Vacher, Secretaire General, c/o Institut National de Medecine Agricole, Tours (Indre-et-Loire), France.

• • • Fourth International Conference on Bio-Medical Electronics. New York, July 9-14, 1961. Herman Schwan, School of Electrical Engineering, University of Pennsylvania, Philadelphia, Pa.

• • • Sixth International Nematology Symposium. Ghent, Belgium, July 25-28, 1961. Dr. O. Ahlberg, Statens Vaxtskyddsanstalt, Solna 7, Sweden.

• • • First International Congress of Biophysics. Stockholm, July 31-Aug. 4, 1961. Dr. Bo Lindstrom, Department of Medical Physics, Karolinska Institutet, Stockholm 60, Sweden.

• • • Fourteenth International Union of Biological Sciences. Amsterdam, July, 1961. Prof. G. L. Stebbins, Secretary-General, c/o Department of Genetics, University of California, Davis, Calif.

• • • Fifth International Congress of Biochemistry. Moscow, Aug. 10-16, 1961. Dr. N. M. Sissakian, Secretary-General, Leninsky prospekt 33, Moscow, B-71, U.S.S.R.

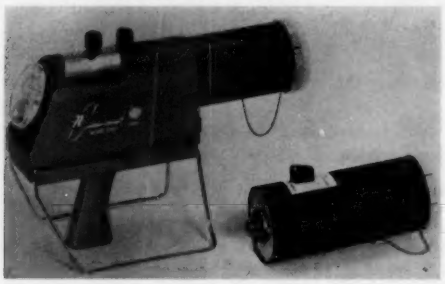
(continued on page 624)

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave, Chicago 5, Illinois.

Integrating-Rate Survey Meter

The first gun-type survey meter providing both rate of dose and accumulated dose measurements is being manufactured by Nuclear-Chicago Corporation. The instrument is used for monitoring beta-, gamma-, and x-radiation of low and medium intensity. It may also be used for measuring alpha-radiation with energies as low as 4.5 mev.

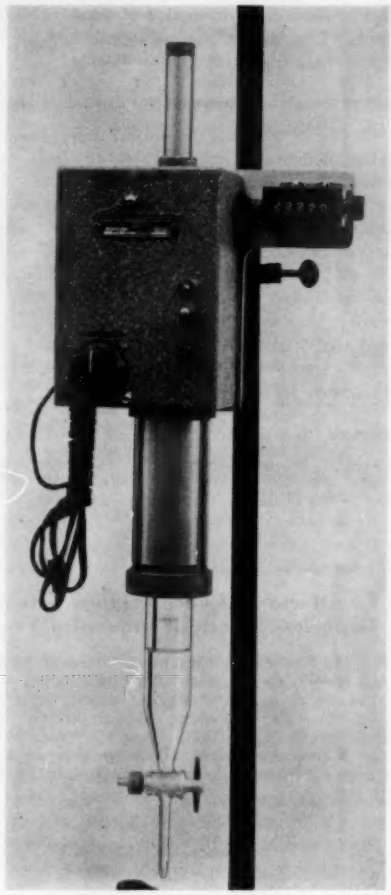


The "rate" and "integrating" chambers supplied with the instrument can be easily interchanged. The "rate" chamber gives measurements in 3 ranges: 0–25, 0–250, and 0–2,500 milliroentgens per hour. The "integrating" chamber gives accumulated measurements in 5 ranges: 0–0.25, 0–2.5, 0–25, 0–250, and 0–2,500 milliroentgens. Energy dependence of both chambers is only ±4%.

The new survey meter can be used in monitoring radiation in radioisotope laboratories, for checking shipping containers, and for room and personnel dosimetry studies. It is ideal for scatter x-ray or gamma measurements.

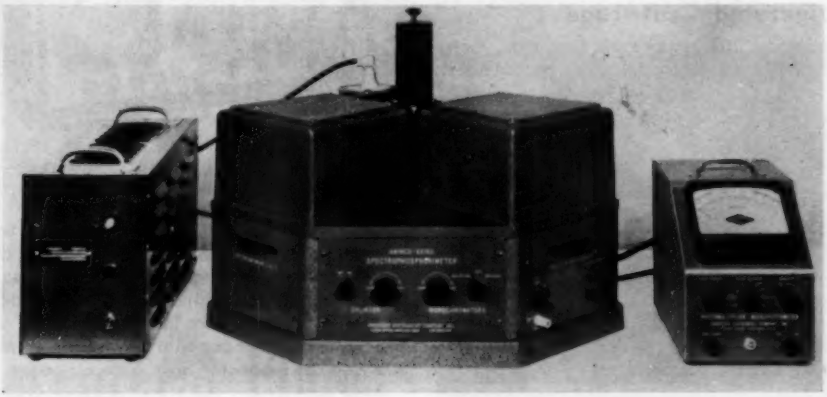
Buret Attachment

A buret attachment which increases titrant reservoir capacity 10 times has been designed by the American Instrument Co. for use with the Aminco-Koegel Menisco-Matic Buret.



The 32.0-ml. volume buret permits analysis to titrate volumes ranging from micro through macro quantities. Because of the precision and accuracy of the buret, concentrated titrants can be used to (1) complete a titration wherein the volume of the titrate remains constant, (2) maximize end point effects irrespective of the method used, or (3) perform up to 35 titrations without refilling the buret. Quantities of titrant required and time to perform the titrations are sharply reduced.

The attachment permits quick and simple conversion of the standard 3.2-ml. buret to the large-volume buret. A stopcock arrangement is incorporated in the reservoir, permitting automatic refill without removal of the reservoir from the buret.



Spectrophosphorimeter

An instrument capable of exciting, measuring, and recording phosphorescence of compounds has recently been introduced by the American Instrument Co., Inc.

The "Spectrophosphorimeter" excites compounds and measures resulting in phosphorescence throughout the ultraviolet and visible regions. Since most organic molecules, hen dispersed in suitable media, produce unique phosphorescences when excited by ultraviolet light, recordings may be used to identify various components in compounds of unknown composition.

The instrument can also quantitatively analyze phosphorescent compounds. Since phosphorescent intensity is directly proportional to concentration, once the intensity of the unknown sample has been measured, work curves can be established from which the unknown concentrations can be identified.

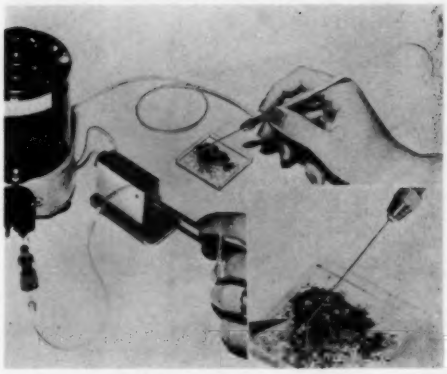
Mean lifetime of the phosphorescent state (decay curve) is measurable using either an X-Y recorder with time base generator or an oscilloscope.

In addition to measuring phosphorescence, the Spectrophosphorimeter will also, with an accessory attachment, measure fluorescence throughout the ultraviolet and visible regions.

Vacuum Pencil

A vacuum pencil has been developed by Kulicke and Soffa Manufacturing Co. for the simple and efficient handling of pieces of metal, paper, glass, biological specimens and other particles as small as 0.015 inch in diameter.

One model is valve-actuated, hand or knee operated, utilizing any of 4 different interchangeable tips. Other types are used with suctions cups and are available with or without vacuum pumps.



Each unit possesses excellent balance and is constructed to prevent slippage from fingers or hand while being used.

Compact Refrigeration Cabinet

A low-temperature refrigeration cabinet that takes up only 7 square feet of floor space is being manufactured by Alpha Electric Refrigeration. Available in seven low temperature ranges, it can be used to store pharmaceuticals or semen as used in artificial breeding.

Mechanically refrigerated and self-contained, the cabinet is easily serviceable. The durable hermetic units may be shut down without opening or closing valves and without danger of losing refrigerants.

Refrigerated Centrifuge

The Model 20R refrigerated centrifuge, manufactured by Labline, Inc., is designed for testing materials at room temperature to as low as −20C. A speed of 19,000 r.p.m. can be attained with

high-speed attachments. An electric brake provides smooth deceleration. The hermetically sealed ¾ h.p. refrigerator compressor and ¾ h.p. motor (sealed lubrication) are cushioned to eliminate vibration and minimize noise. The unit accommodates all angle and swinging heads normally used on nonrefrigerated size 1 and 2 centrifuges, and all makes and sizes used on refrigerated centrifuges.

Storage space for accessories is provided in the base of the cabinet.

Spectrometer

A transistorized spectrometer, or "nuclear brain," which automatically distinguishes between background radiation and radioactivity of the object being analyzed, has been developed by the Victoreen Instrument Co. The new spectrometer has 200 data storage channels with total capacity of 20 million "counts," making it especially advantageous for comparing analyses of several samples. The radiation count of the substance being studied and the count of normal background radiation may

be fed to the spectrometer simultaneously. It then automatically subtracts the background radiation while adding the study count, assuring true qualitative analysis of the sample. Information can be read during the test by means of an analog display or neon digital indicators. Stored data can be called out through a print-out on a digit printer, a standard read-out typewriter, or through a plot-out.

External-Internal Defibrillator

A new Morris external-internal defibrillator, developed under contract with the U. S. Air Force, is being manufactured by Corbin-Farnsworth Inc. The new instrument permits full utilization of trans-thoracic cardiac massage, the new technique which avoids patient shock.

Four and three output-voltage settings are provided for open-chest and closed-chest defibrillations. Impulse durations are automatically timed and are

independent of the length of time the defibrillation push button is held down.

Insulated electrodes, an isolation transformer for the output, a circuit breaker for the input power, and keyed output terminals to prevent inadvertent electrode connections are safety features of the new design. No warmup period is required to put the model into operation.

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Correspondence

February 8, 1961

Dear Sir:

I am writing to protest a statement contained in the summary of an article entitled "The Isolation of Infectious Bovine Rhinotracheitis Virus from Cattle Affected with Conjunctivitis—Observations on the Experimental Infection" by Doctors Abinanti and Plumer, appearing in the January, 1961, issue of the *American Journal of Veterinary Research*.

The statement which I find objectionable is the second sentence in the summary:

> "Clinically, it simulated keratoconjunctivitis or 'pink eye' but there were no corneal opacities or ulcers."

This statement I consider to be contradictory to the comments made in the third paragraph of the inner column on page 14:

> "Casual clinical observation of this occurrence of conjunctivitis might have classified it as bovine infectious keratitis (BIK). However, the disease differed from BIK in several obvious characteristics. It was primarily an infection of the ocular and palpebral conjunctiva. The cornea was not consistently involved and, when it was, the changes were mild, did not include ulcers, and were confined to the limbus. The discharge was primarily serous in character."

My objection is twofold; first and most important, is the fact that *Moraxella bovis* is conceded by practically all investigators of infectious keratoconjunctivitis to be its cause. Statements such as are made in the summary cast a question on the cause of infectious keratoconjunctivitis. It is unfortunate bacteriological examinations were not made in the course of this work and that the authors chose to compare the IBR conjunctivitis with a specific disease entity of established etiology.

My second objection is of less importance. You will notice in the second quote cited the phrase "*Casual* clinical observation . . . " is used along with the term, "might"—I question the use of such loose terminology since it is completely irrelevant to factual research reporting. If it has a place, it belongs under the heading, "Discussion."

In closing, I wish to state that I consider the publication to contain valuable information of importance to veterinary medicine. My objections as stated above are based entirely upon the manner in which the data was presented.

Very truly yours,
S/EARL M. BALDWIN, JR., D.V.M.
Omaha, Nebraska

February 23, 1961

Dear Sir:

The words "casual" and "might" were purposely used by the authors as a warning for clinicians to aid them in arriving at a more definite diagnosis. I have reread the article "The Isolation of Infectious Bovine Rhinotracheitis Virus from Cattle Affected with Conjunctivitis—Observations on the Experimental Infection," and fail to find statements which might lead one to infer that infectious keratoconjunctivitis was caused by IBR virus. I believe Dr. Baldwin would agree that were a vaccine available for "pink eye" no statements made in this paper would prejudice its use in favor of IBR vaccine.

No objection can be found to the wish that bacteriologic studies might also have been done; however as shown in the paper, the experimental disease was similar to that seen in the field and these calves were infected with bacteriologically sterile tissue culture fluids.

Sincerely yours,
S/FRANCIS R. ABINANTI, D.V.M., PH.D.
Bethesda, Md.

MEETINGS—Continued

• • • First International Pharmacological Meeting. Stockholm, Aug. 22-25, 1961. Dr. Arvid Wretlind, Secretary General, Karolinska Institutet, Stockholm 60, Sweden.

• • • American Institute of Biological Sciences. Lafayette, Indiana, Aug. 27-Sept. 1, 1961. John R. Olive, Assistant Executive Director, 2000 P. Street N. W., Washington 6, D.C.

• • • Eighth Congress of the European Society of Haematology. Vienna, Aug. 28-Sept. 2,1961. Dr. H. Fleischhracker, Secretary General, Frankgasse 8, Billrothhaus, Vienna IX, Austria.

• • • International Congress of Exfoliative Cytology, Vienna, Aug. 31-Sept. 2, 1961. Office of the Secretary of the Congress, 666 Elm St., Buffalo 3, N.Y.

• • • Seventh International Congress of Biological Standardization. London, Aug. 28–Sept. 1, 1961. E. C. Hulse, Secretary of Organizing Committee, Ministry of Agriculture, Central Veterinary Laboratory, Weybridge, Surrey.

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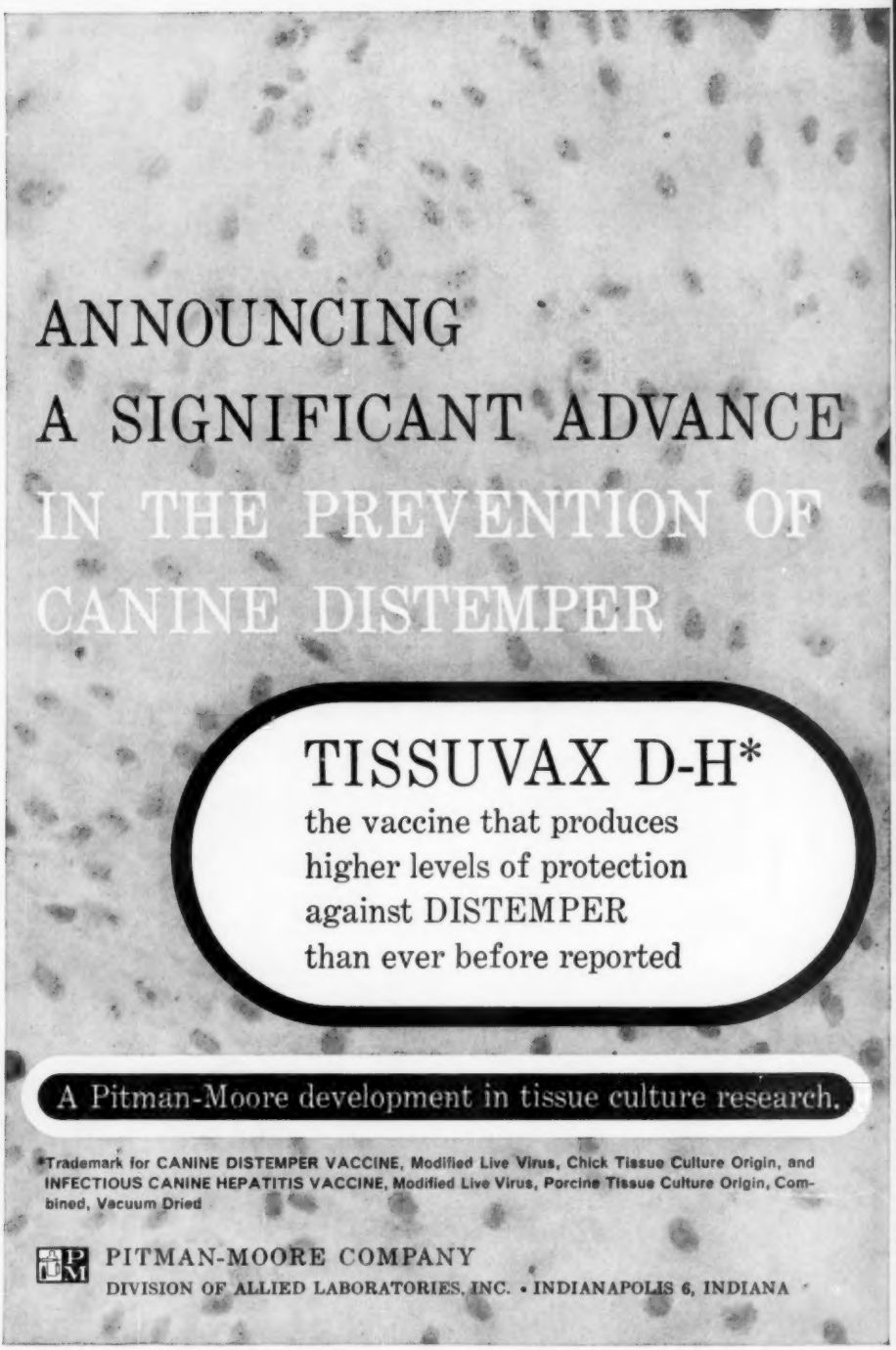
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